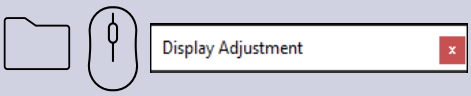
# IMARIS TRAINING

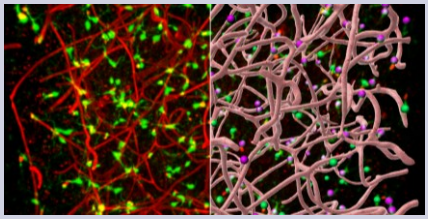
OXFORD INSTRUMENTS | IMARIS

## Imaris environment and navigation




## Microglia cells count & attraction to the vasculature

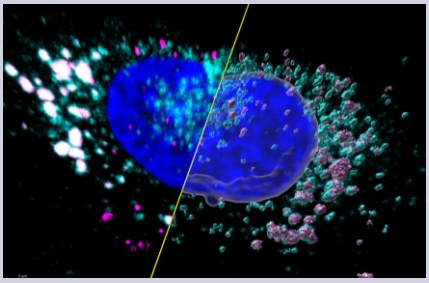
### Exercise 1 - 45 mins



**Question** : Are microglia cells attracted to the vasculature ?
**Topics** :
Cells quantification with Spots
Cells attraction band identification
Cells classification according to the distance to another reference structure detected as « Surface »

## Colocalization & intensity profile around nuclei

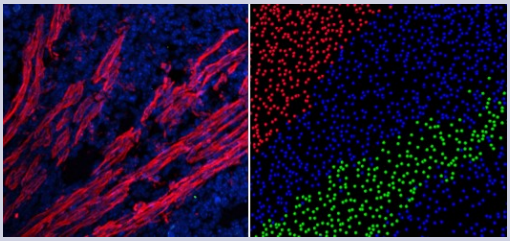
### Exercise 2 - 20 mins



**Question**: Are specific vesicles more likely to be close to the nucleus membrane ? Do my two sets of vesicles colocalize? If yes, to which extent ?
**Topics**:
Cells quantification with Surfaces
Intensity profile
Colocalization measurement using Surfaces overlapping regions

## Cell density & classification per region

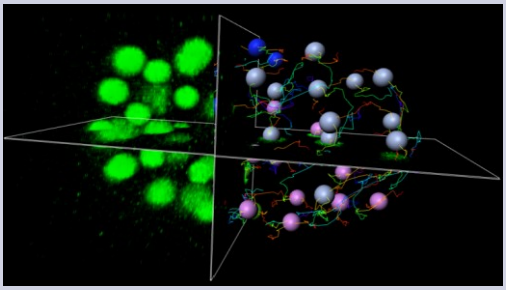
### Exercise 3 - 15 mins



**Question:** Measure cell densities over different regions of interests not defined by a specific staining ?
**Topics**:
Classification of cells as Spots per regions using Machine Learning
Cell density measurements

## Changes in cell speed & intensity over time

### Exercise 4 - 30 mins



**Question:** How cell dynamics and intensities vary over the cell cycle?
**Topics:** Cells tracking with Spots
Spots Tracks manual labelling
Automatic cells dynamics analysis before and after the cell splitting event

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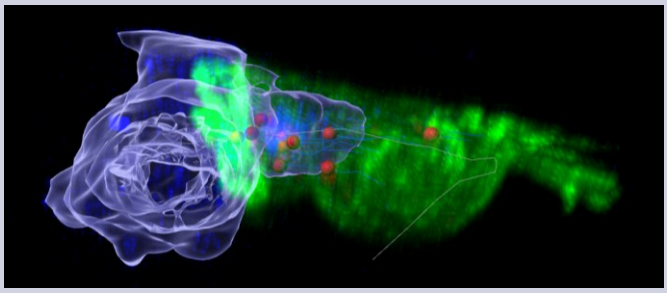
## Killer T cell dynamic interacting with a cancer cell

### Exercise 5 - 10 mins

**Question:** How do cytotoxic granules behave when immune killer cells contact a cancer cell?
**Topics:**
Granules speed analysis before and after the moment cells contact
Define an Image Time Event manually

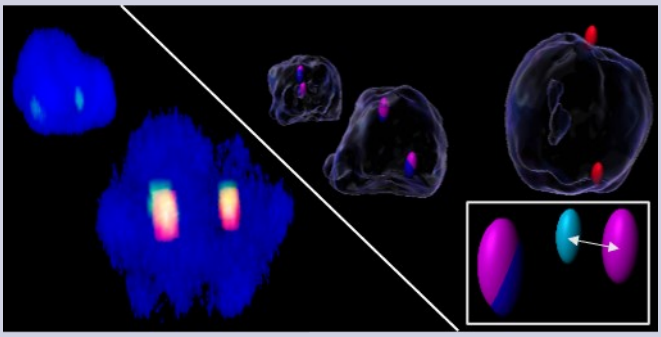


## Batch 3D genomic analysis by DNA FISH

### Exercise 6 -45 mins

**Question:** Do two types of alleles colocalize and if yes to which extent? Analysis to be repeated and normalized on multiple images.

**Topics:**
On multiple folders of multiple images: batch alleles detection using Spots, batch alleles colocalization using inter-object distances
Visualize results in an Imaris plot
Extract statistics results for further analysis

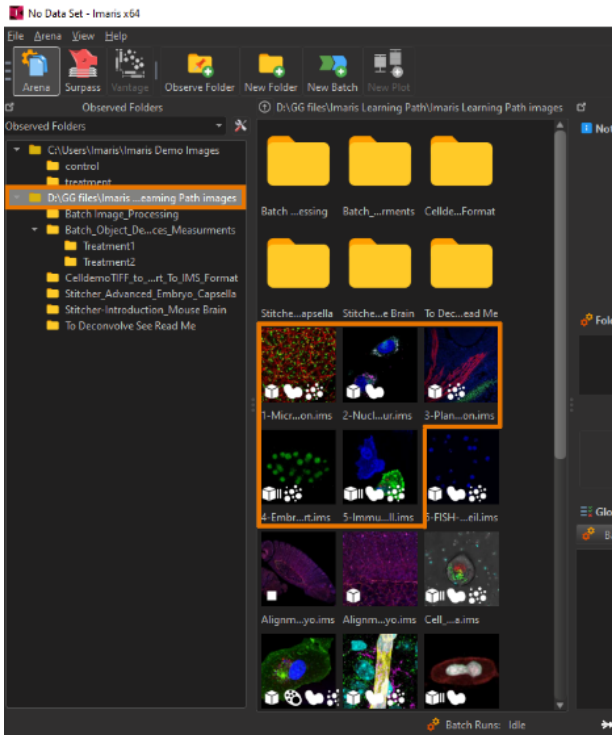


# Imaris environment and navigation

**Environment**: to get familiar with the Imaris, take this Imaris tour video (7mins30s)

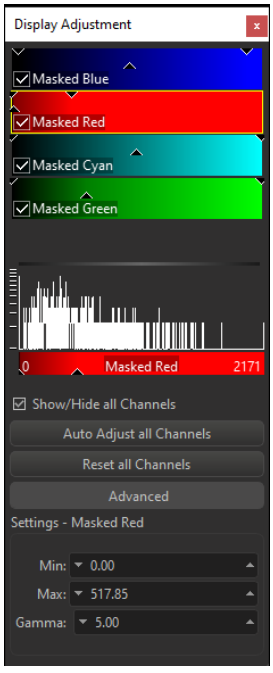
### Image files

All **image files** used in the video tutorials listed in the Imaris Learning Path and in this Imaris Training documents can be accessed from the **Arena** interface in the **Imaris Learning Path Folder**



### Display Adjustment

If you don't see the Display Adjustment window then Click on Edit/Show Display Adjustment or **CTRL+D**

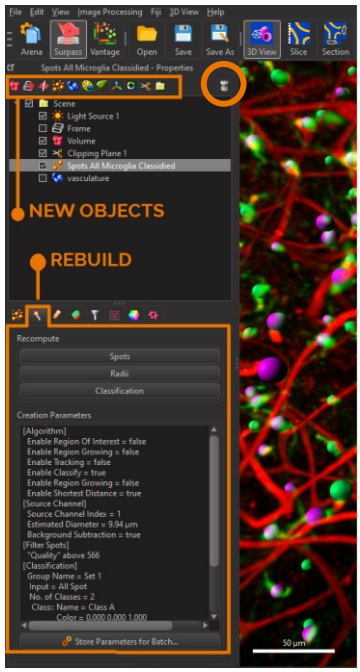



### Navigation and selection

- Left click and Drag to **rotate** the image
- Right Click and Drag to **Translate** the image
- Middle button to adjust the **zoom**
- Alt-key to **force navigation**, you can't select/modify any objects while you hold Alt
- Mouse **Object Select** Mode: the first click selects the object and the second click lets you modify/move it

### Add, Rebuild, Delete objects

- You can **Add** a new object. The object creation wizard starts from the beginning. All parameters shown will be default values
- You can **Rebuild** existing objects. The object creation wizard from the beginning. All parameters shown will be the ones chosen during the previous building process. They won't be the default values
- You can **Delete** existing objects: Highlight the object and then click on the bin icon circled in orange below



# Microglia cells count & attraction to the vasculature

## Exercise 1 - 45 mins

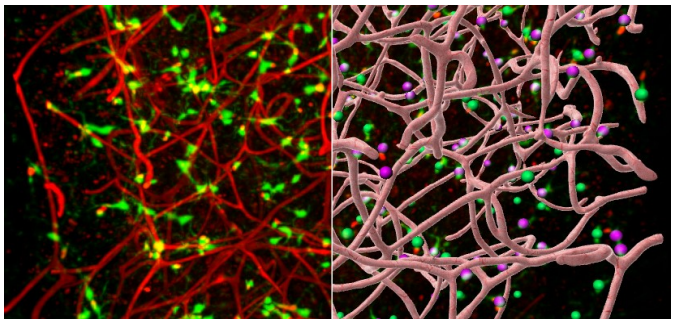
### Dataset description

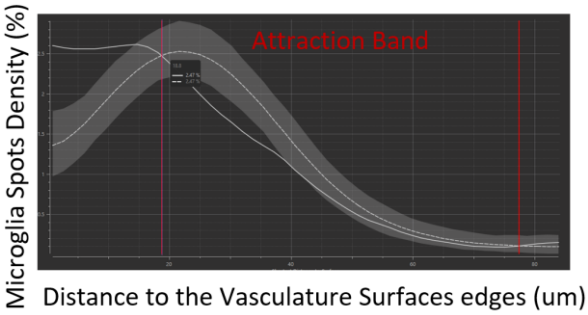
**File: 1-Microglia-Vasculature_Kofron.ims**
Channel 1 : Microglial cells (Green)
Channel 2 : Vasculature (Red)

Collected with a multiphoton, 25X silicon immersion lens. Sample approx. 400um deep section of adult mouse brain. Courtesy of Dr. Matthew Kofron, Confocal Imaging core, Cincinnati Children's Hospital Medical Center, OH USA

### Scientific question and complete image analysis protocol

**Are microglia cells attracted to the vasculature?**
1- Model Microglia cells as Spots to count them in Ch1 Green
2- Model the Vasculature as Surfaces to detect it in the Ch2 Red
3- Identify the "Attraction Band" where Microglia Spots are attracted by the Vasculature
4- Create 2 subpopulations of microglia and label them according to their positions in/out of the Attraction Band

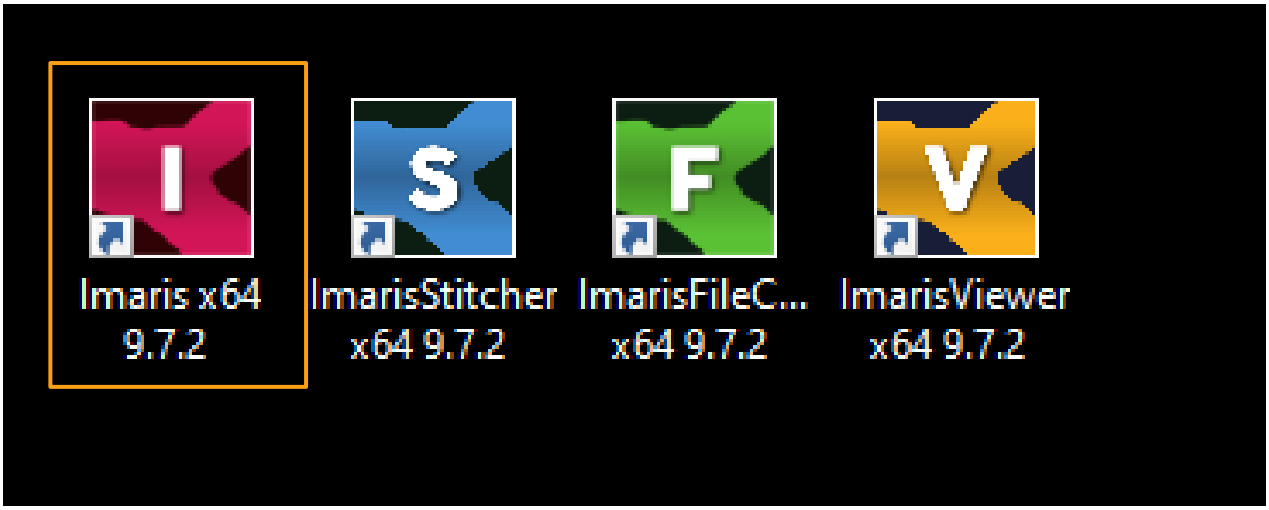




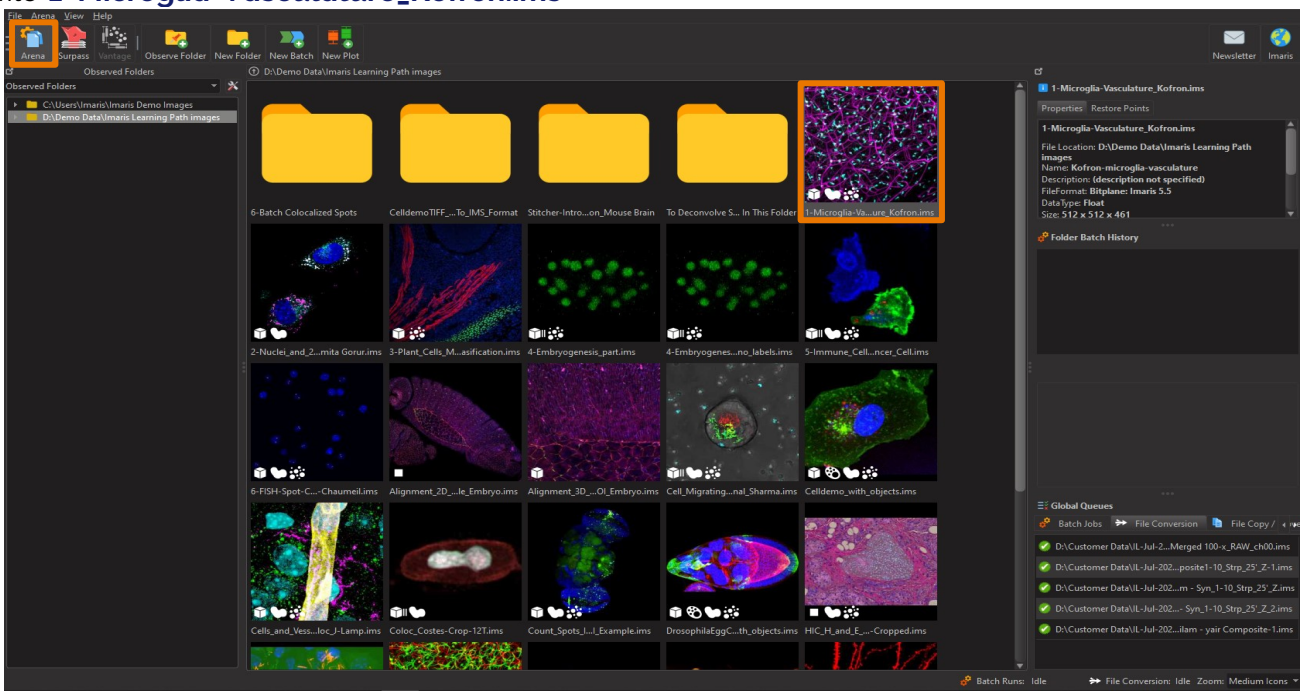

### Step by step guided exercise

#### Open the image file:

Open Imaris by double clicking on the main Imaris pink application icon:

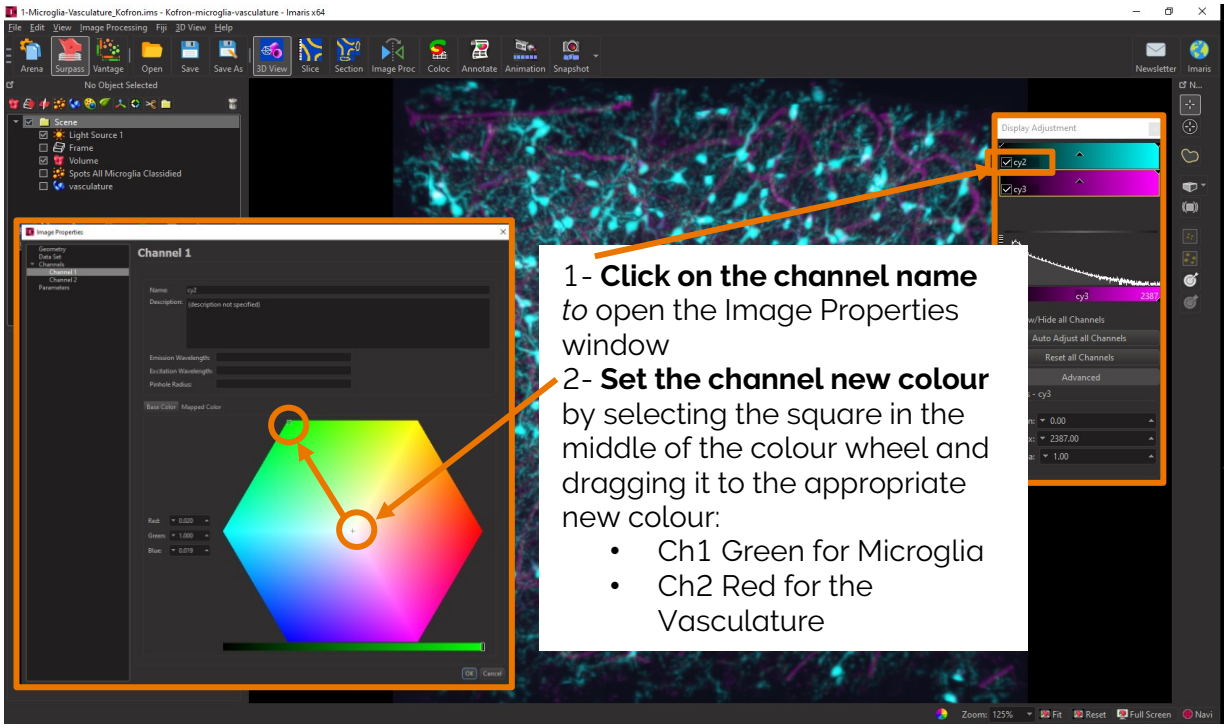


Make sure you are in the "Arena" interface which is Imaris library of images and double click on the file **1-Microglia-Vasculature_Kofron.ims**

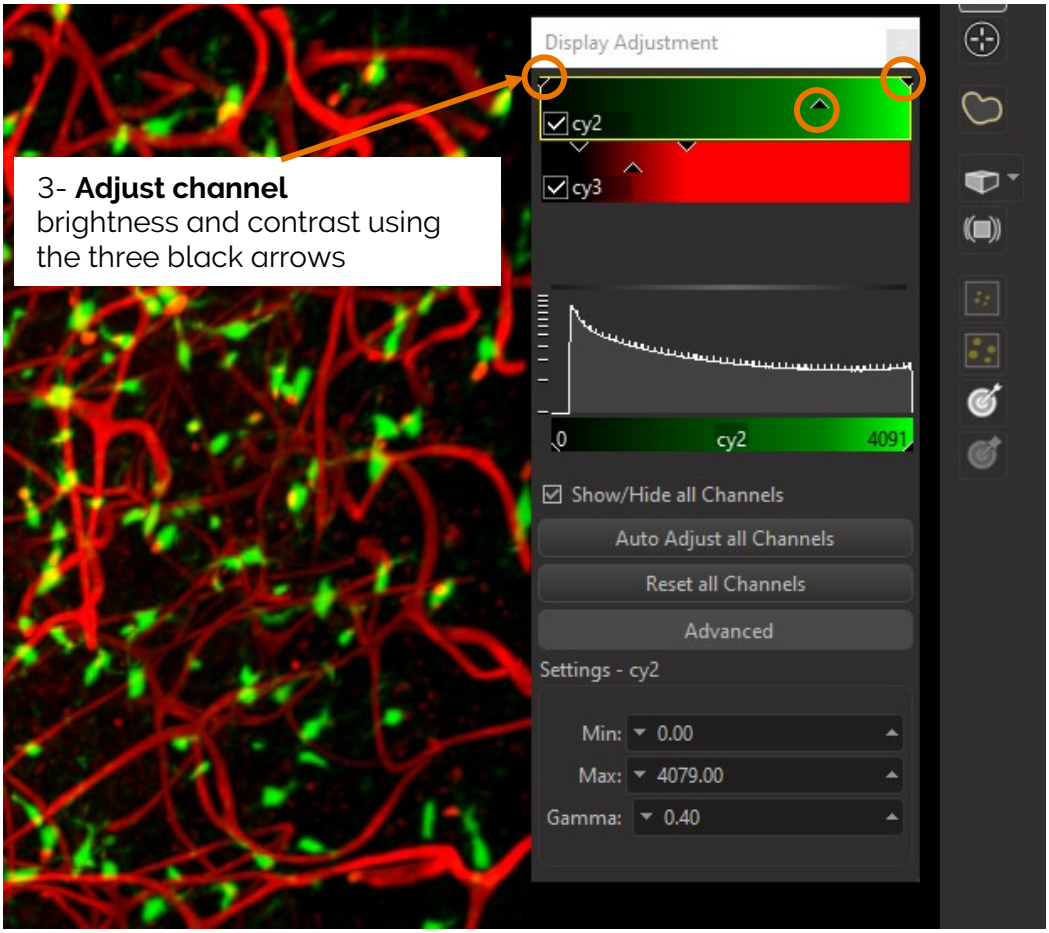


## Channel Display Adjustment

Adjust the Channel renderings for optimal visualisation:

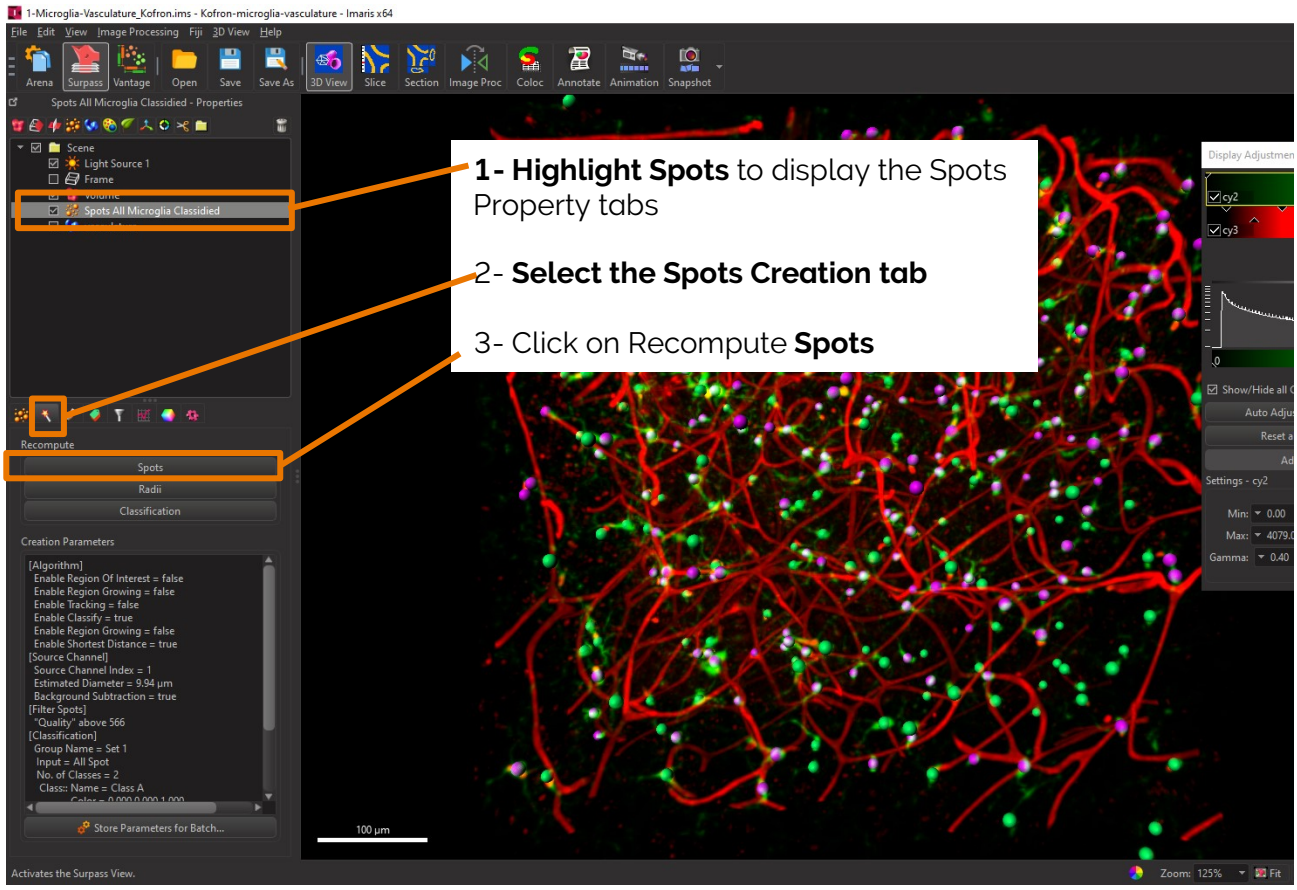



For more information on the Display Adjustment window Click this button 

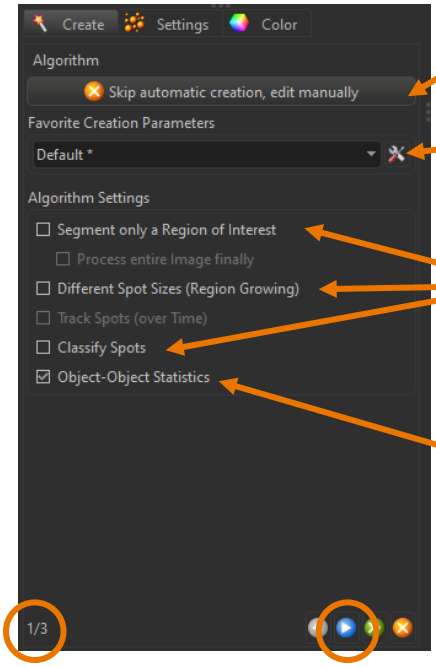



## Spots Creation Wizard: Rebuild

Rebuild the existing Spots object to count Microglia and get their positions



## Spots Creation Wizard: Step 1/3




**Don't click,** we'll generate Spots automatically

**No parameters to load,** as we are rebuilding the existing Spots, parameters previously chosen will be shown

**Uncheck:** we'll work on the full image, create Spots of the same size ( we only need count & positions) and we'll classify them later

**Check** as we'll need the distance from the Microglia Spots centres to the Vasculature Surfaces edges

➤ Click on the **Blue arrow** circled to go to the next step

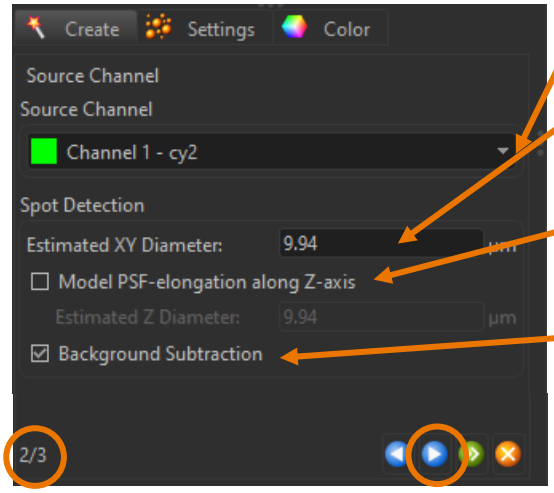
Option explanations:

- **Skip automatic creation, Edit manually**: if clicked, you'll skip all steps of the automatic creation wizard and you will have to create Spots manually.
- **Favourite creation parameters**: you can load previously saved parameters as favourite.
- **Segment only a Region Of Interest**: if checked, during the set up and optimisation process of the wizard of creation parameters, calculation will be restricted to an ROI so that it is fast. Once all steps of the wizard of creation are completed, calculation will run on to the full image. Here, we uncheck this box as we are working on a demo image however once you'll be working on your own dataset, best is to check it to gain in speed.
- **Different Spot Sizes**: This is to create Spots with different diameters adjusted to the signal. If it is not checked then all created Spots will have the same diameter. Here, as we just need Spots count & positions and considering that all Microglia have very similar cell bodies (in terms of size and intensity), Spots of the same sizes will do.
- **Classify Spots**: if checked, once Spots are detected, it enables automatic Spot Classification using either 1D or 2D filters or Machine Learning.
- **Object-Object statistics**: if checked, it enables the calculation of distances between objects.

➤ **Blue arrow**: to move from one step to the next one or previous one

➤ **Green arrow**: to finish the wizard of creation automatically using either default or loaded favourite or previously used parameters

## Spots Creation Wizard: Step 2/3




Select **Channel 1** Green from the drop-down as it has the Microglia cell bodies

**Type in around 10 um,** Microglia cell bodies rough diameter. (Click on the ruler icon to learn how to measure )

**Don't check**

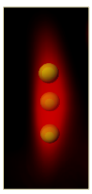
**Check Background Subtraction**

Click on the **Blue arrow** circled to go to the next step

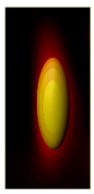
Option explanations:

- **Source Channel:** Select the channel containing the structures you want to detect as Spots.
- **Estimated Diameter;** Type in an estimation of the diameter of your typical structures. Objects with size smaller than the specified Estimated Diameter are not detected.
- **Model PSF-elongation along Z-axis:** This is to create elliptical shaped Spots in opposition to spherical shaped Spots which is the default. This parameter specifies the dimension of the Spots object in the Z direction (usually 2 x XY diameter). This is useful for images where the signal is largely stretched in the Z direction like in Wide Field images not deconvolved. It can correct for artefacts as shown below:

Volume Rendered Object



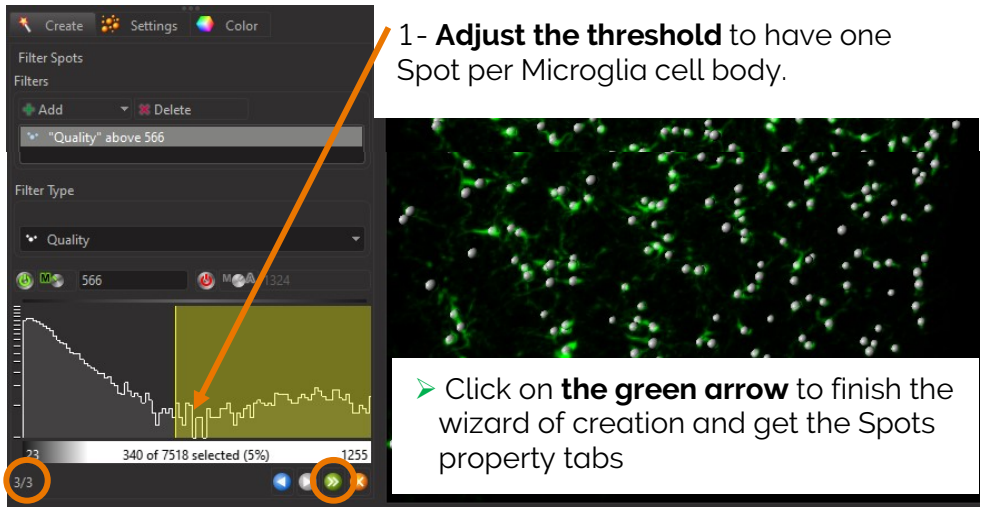
Spherical Spots (artifact)



Ellipsoid Spots (correct)

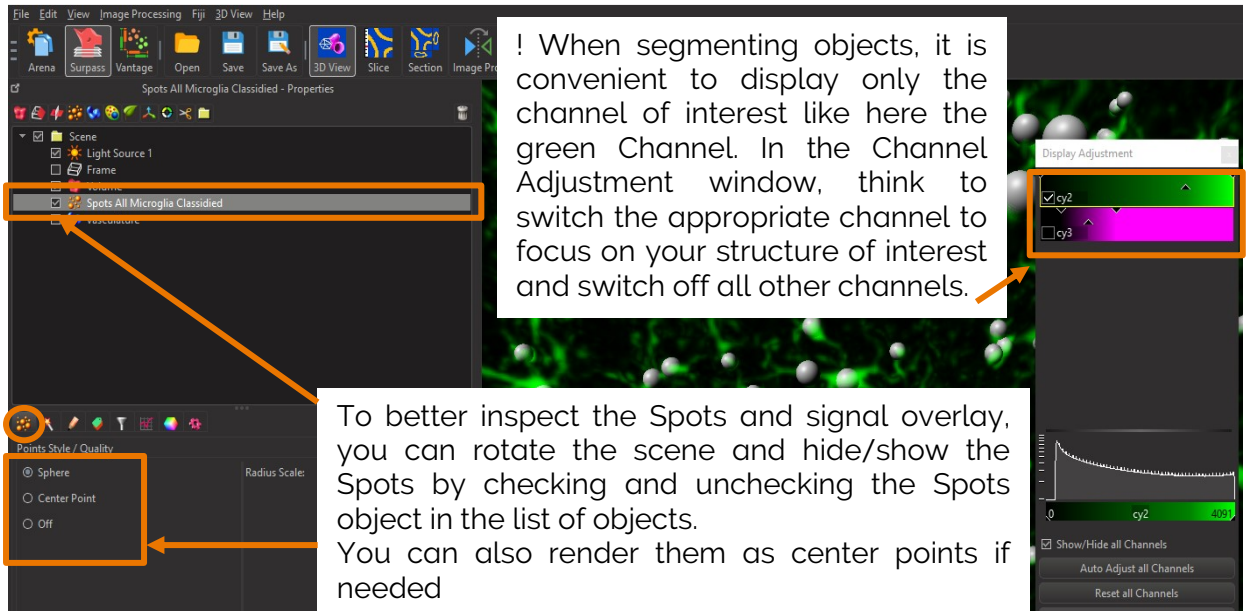
- **Background Subtraction:** the background will be removed prior to Spots' detection. Technically background subtraction smoothes the image. The intensity is the one of a Gaussian filtered channel (Gaussian filtered by 3/4) minus the intensity of the original channel Gaussian filtered by 8/9 of sphere radius.

## Spots Creation Wizard: Step 3/3




1- **Adjust the threshold** to have one Spot per Microglia cell body.

➢ Click on **the green arrow** to finish the wizard of creation and get the Spots property tabs




! When segmenting objects, it is convenient to display only the channel of interest like here the green Channel. In the Channel Adjustment window, think to switch the appropriate channel to focus on your structure of interest and switch off all other channels.

To better inspect the Spots and signal overlay, you can rotate the scene and hide/show the Spots by checking and unchecking the Spots object in the list of objects.
You can also render them as center points if needed

Option explanations:

**Histogram**: In the histogram the regions above and below the threshold values are displayed in white, while region within threshold is displayed in yellow

- The green A stands for Automatic threshold
- The green M stands for Manual threshold
- Left click to move the lower threshold
- Right click to move the higher threshold
- Scroll to zoom in the histogram
  The histogram has a zoom bar at the top:
  - Mouse wheel allows to zoom in and out.
  - Holding right-click on the zoom bar allows to pan.
  - Double right-click resets the zoom.

**Spots filtering** is incorporated into the creation process. Filters allow the removal of objects that would interfere with interpretation of results.

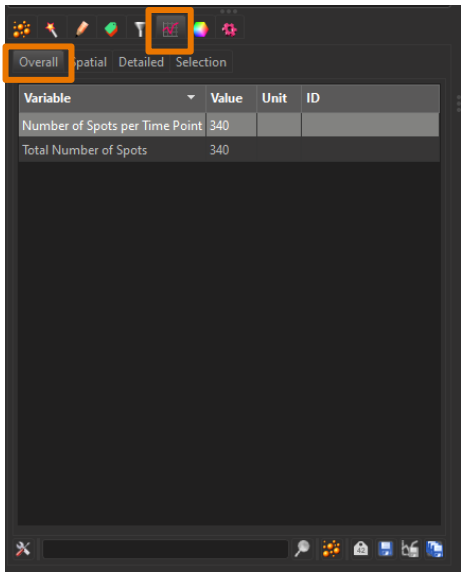
**Quality**: The 'Quality' is the intensity at the centre of the spot in the channel Spots were detected.

- If 'Background Subtraction' was not checked this is the intensity of the channel Gaussian filtered by 3/4 of spot radius.
- If 'Background Subtraction' was active the intensity is the one of a Gaussian filtered channel from above minus the intensity of the original channel Gaussian filtered by 8/9 of spot radius.

## Spots Statistics

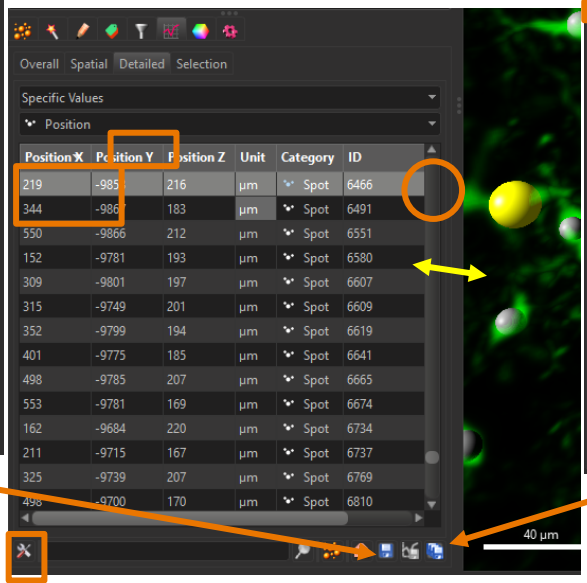
The Spots creation wizard turns into a list of Property tabs ( click  to explore all properties) of which the Statistics tab:

**Overall Spots Statistics:**
Total number of Spots.
Here 340 (Microglia)

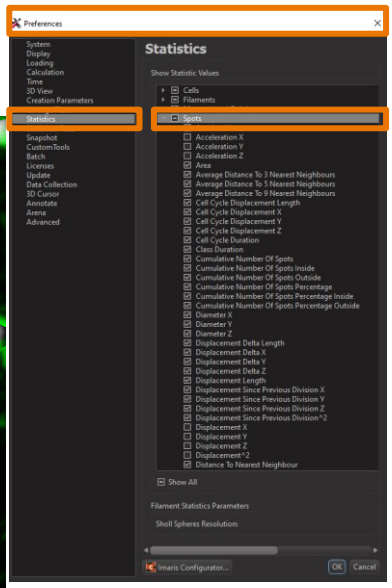



**Detailed Spots Statistics:**

- Here, **Position** was selected from the drop-down menu circled in orange
- In the table, **selecting a line, selects the associated spot** (in yellow) in the 3D scene and vice versa




**Preferences window**




- **Exports** only the **observed statistic** in the table

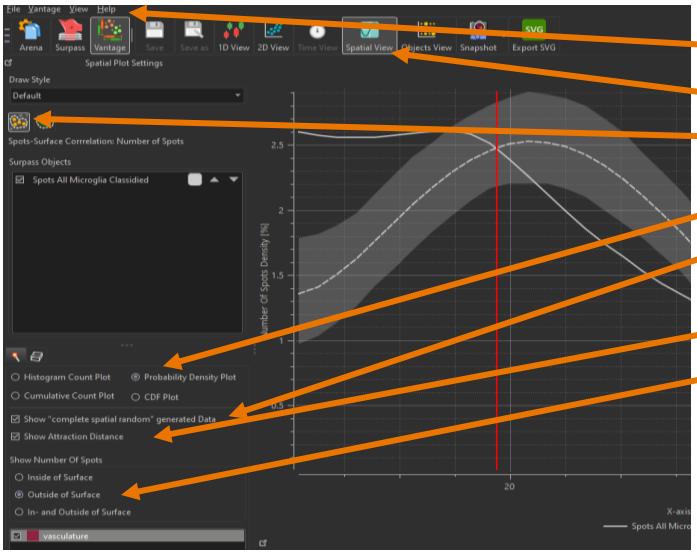
**Exports all** calculated **statistics** as preformatted .xls, .csv or xml file

**Opens** the **Preferences window** under the Spots Statistics where you can select or deselect any Statistic

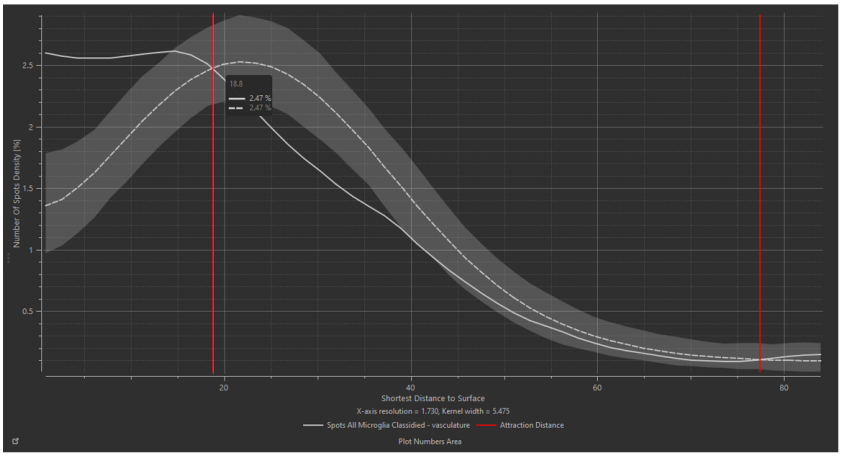
⇨ **Here, we detected all Microglia, got their total count (340) and their positions**

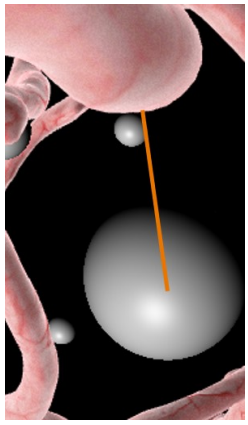
## Attraction Band plot

Create a graph showing the "Attraction Band" of Microglia to the vasculature.
n this exercise, the vasculature was already modelled as a Surface object. To learn how to create a Surface object you can refer to our video tutorials or exercise 2 of the present document.




1- Switch to the **Vantage View**
2- Select **Spatial View**
3- Select **Spots-Surfaces correlation: Number of Spots**
4- Select **Probability Density Plot**
5- Check **Show "complete spatial random" generated data**
6- Check **Show Attraction Distance**
7- Select **Outside of Surface**



- **X axis**: **Distance from the centre of each Microglia Spot to the edge of the closest vasculature Surface.** Positive numbers correspond to Spots outside the Vasculature and negative values to Spots inside the Vasculature. Spots inside the Vasculature could be quantified if the option "Shown Number of Spots" had either been set to **Inside Surface** or **In- and Outside Surface**. Here as the option was set to **Outside Surface** there are only positive values

- **Y axis**: **Microglia Spots Density Percentage**

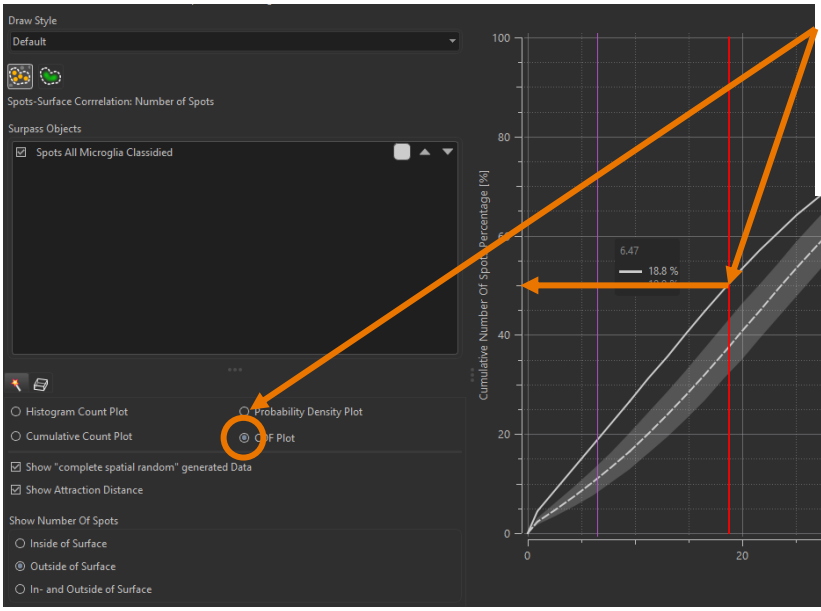
- **White line: Measured Microglia Spots**

- **White dashed line: Expected Percentage of Random Spots**

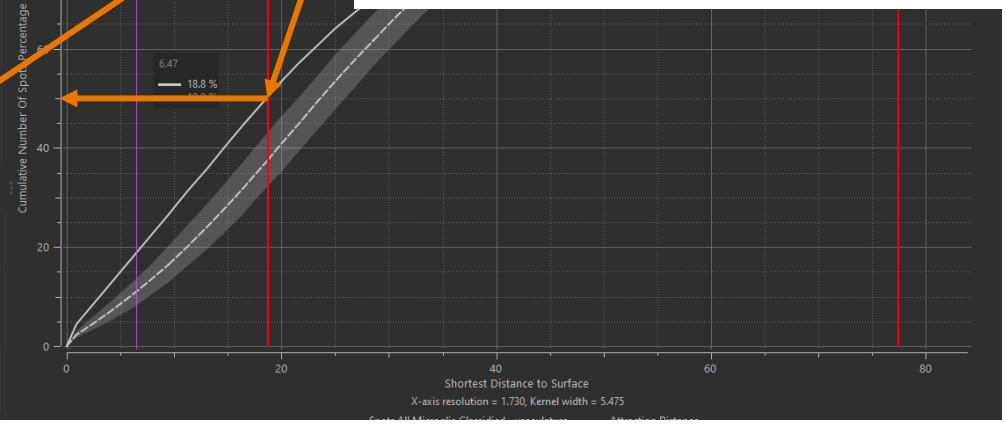
- **Red vertical line: Attraction Distance**

An **attraction or repulsion can be concluded** if the observed (solid line) is outside the confidence interval band of the randomised distribution (dashed line)

⇨ **In this experiment, the attraction band here is of 18.8 um from the edge of the Vasculature Surface. This number is determined by the intersection of the measured Spots curve (white line) and the random (dashed line).**




From the CDF plot, we can see that 50% of the Spots are within the attraction band distance
and this is more than the expected random number (dashed line)



**Spatial statistics** are calculated during surfaces and spots object creation. In this analysis the cumulative number of spots within a distance (d) from the surface are measured, in addition to, the number of spots at a distance (d) from the surface (Not shown in this exercise).
Imaris also simulates randomly positioned spots (the same number as in the image) and generates a confidence band around the expected value of the 1000 simulations, facilitating a useful comparison with the measured object statistics.

**The probability density plot** applies a kernel smoothing width to the data displayed in the histogram plot. The kernel width is displayed beneath the chart in the plot display area.

Confidence Band - Simulation Data
The confidence band of the random spots is computed by taking the number of spots in the image and running a thousand simulations to generate the simulated envelope such that it encloses 98% of the simulated values. With 1% of the simulations falling outside of the simulated envelope i.e. 1% of the simulations are above and below the envelope respectively.

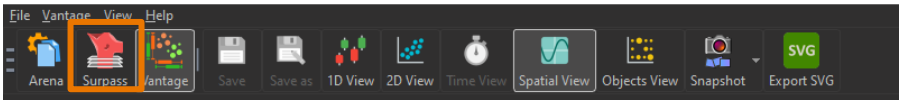
**The methods employed** by Imaris 9.7 are based on well-established methods from the field of spatial statistics. In statistical terminology Imaris 9.7 plots the "intensity" of the observed spots as a function of the shortest distance to the surface, Baddeley 2015 chapter 6.6. Randomization envelopes and hypothesis tests are described in Baddeley 2015, chapter 10. The method was adapted from the approach published by Gomariz et al. 2018: https://www.nature.com/articles/s41467-018-04770-z Gomariz, Alvaro, Patrick M. Helbling, Stephan Isringhausen, Ute Suessbier, Anton Becker, Andreas Boss, Takashi Nagasawa et al. "Quantitative spatial analysis of haematopoiesis-regulating stromal cells in the bone marrow microenvironment by 3D microscopy." Nature communications 9, no. 1 (2018): 1-15

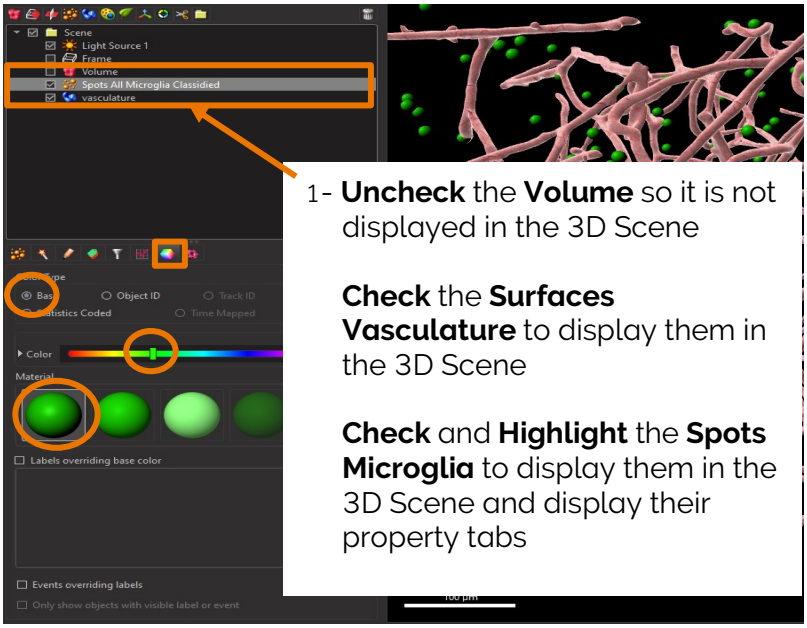
## Spots Microglia 1D Filter Classification

We'll create 2 Microglia Spot classes as follow:

- Spots **in the "Attraction Band"** at a distance **<20um** from the Vasculature Surface edges
- Spots **away from the "Attraction Band"** at a distance **>20um** from the Vasculature edges

First switch back to the Surpass View :

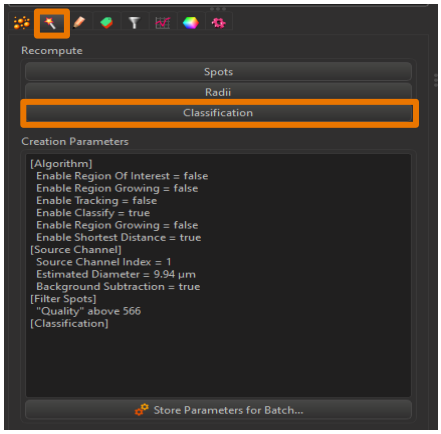


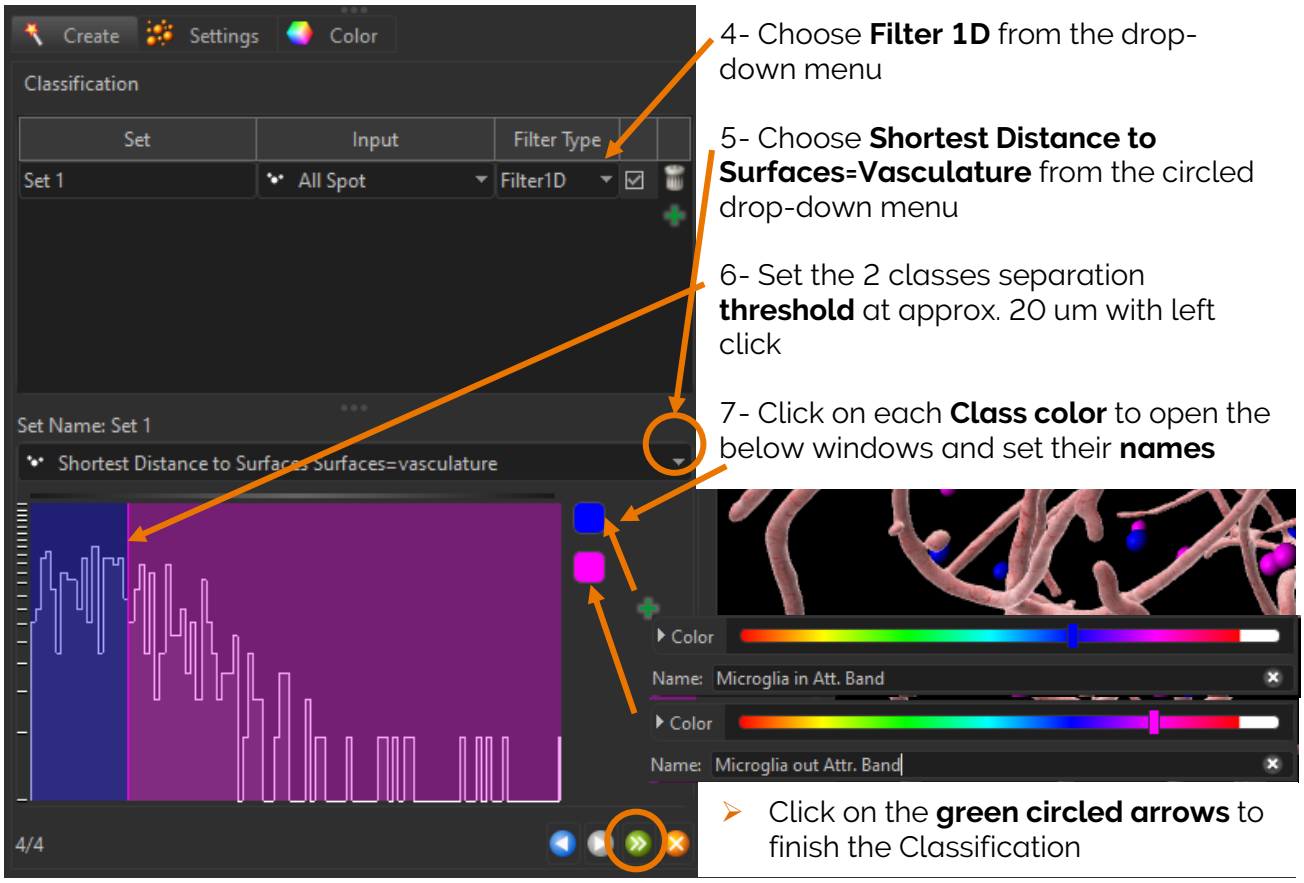

1- **Uncheck** the **Volume** so it is not displayed in the 3D Scene

**Check** the **Surfaces Vasculature** to display them in the 3D Scene

**Check** and **Highlight** the **Spots Microglia** to display them in the 3D Scene and display their property tabs




3- Click on the **Creation tab** (magic wand)
4- **Recompute Classification**




4- Choose **Filter 1D** from the drop-down menu

5- Choose **Shortest Distance to Surfaces=Vasculature** from the circled drop-down menu

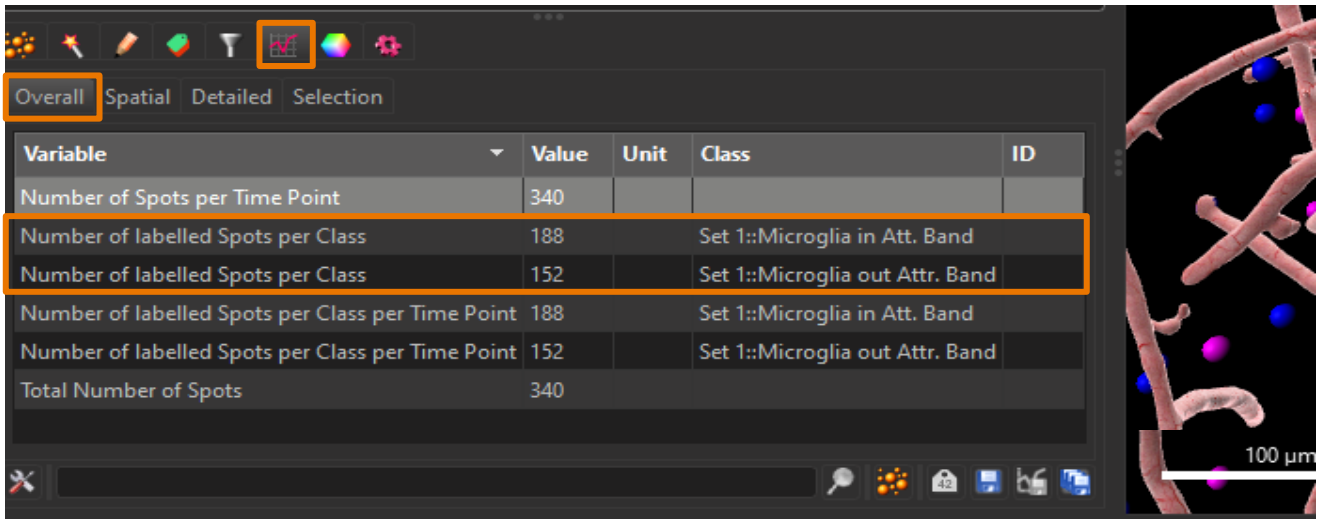
6- Set the 2 classes separation **threshold** at approx. 20 um with left click

7- Click on each **Class color** to open the below windows and set their **names**

➢ Click on the **green circled arrows** to finish the Classification

The Spots creation wizard turns back into a list of property tabs.

⇨ **In the Statistics tab, you can read the total count of Microglia and their counts per class**



## Snapshot

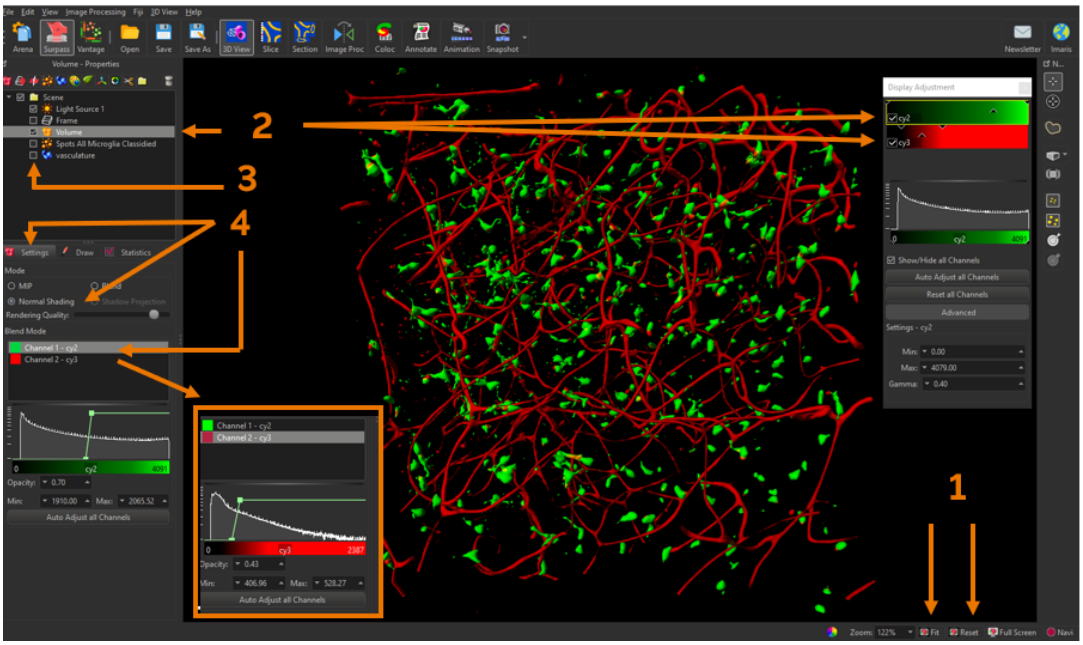
Set the image rendering as follow:

1- Click on **Fit** and then **Reset** to bring the image back to its native position

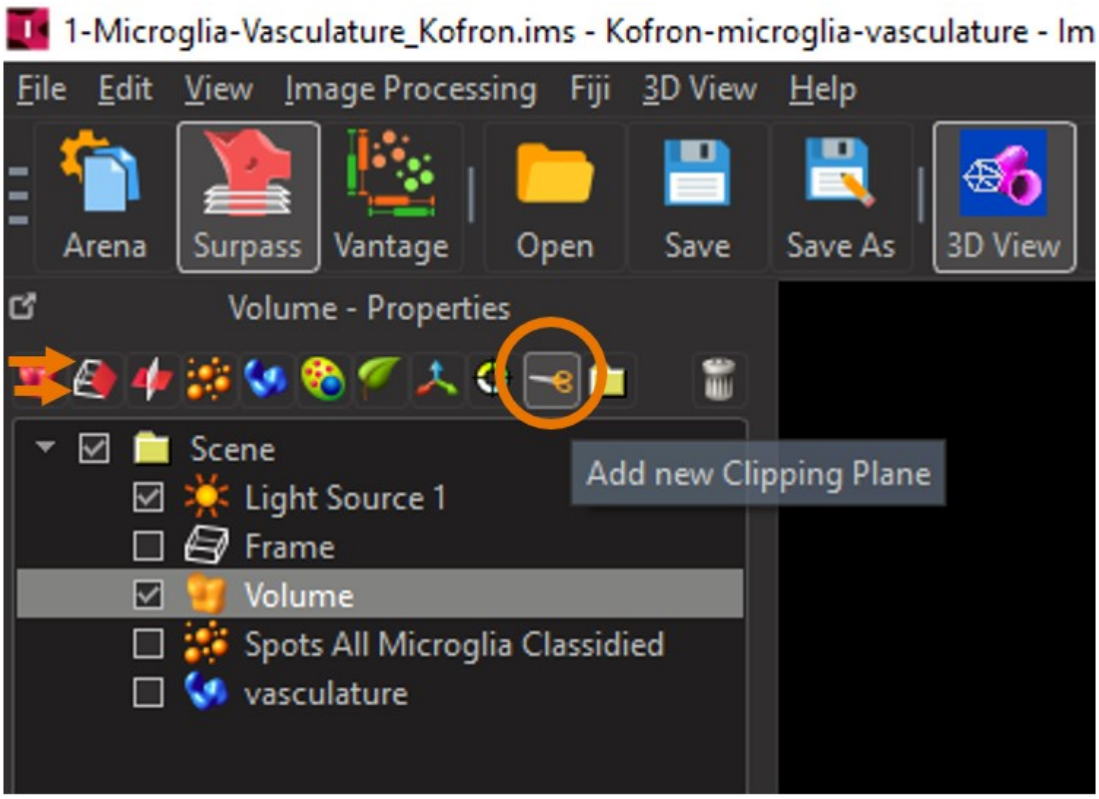
2- **Check & Highlight** the **Volume** and **Check all channels** in the **Display Adjustment** window so they are all visible in the 3D Scene and the Volume Property tabs are displayed

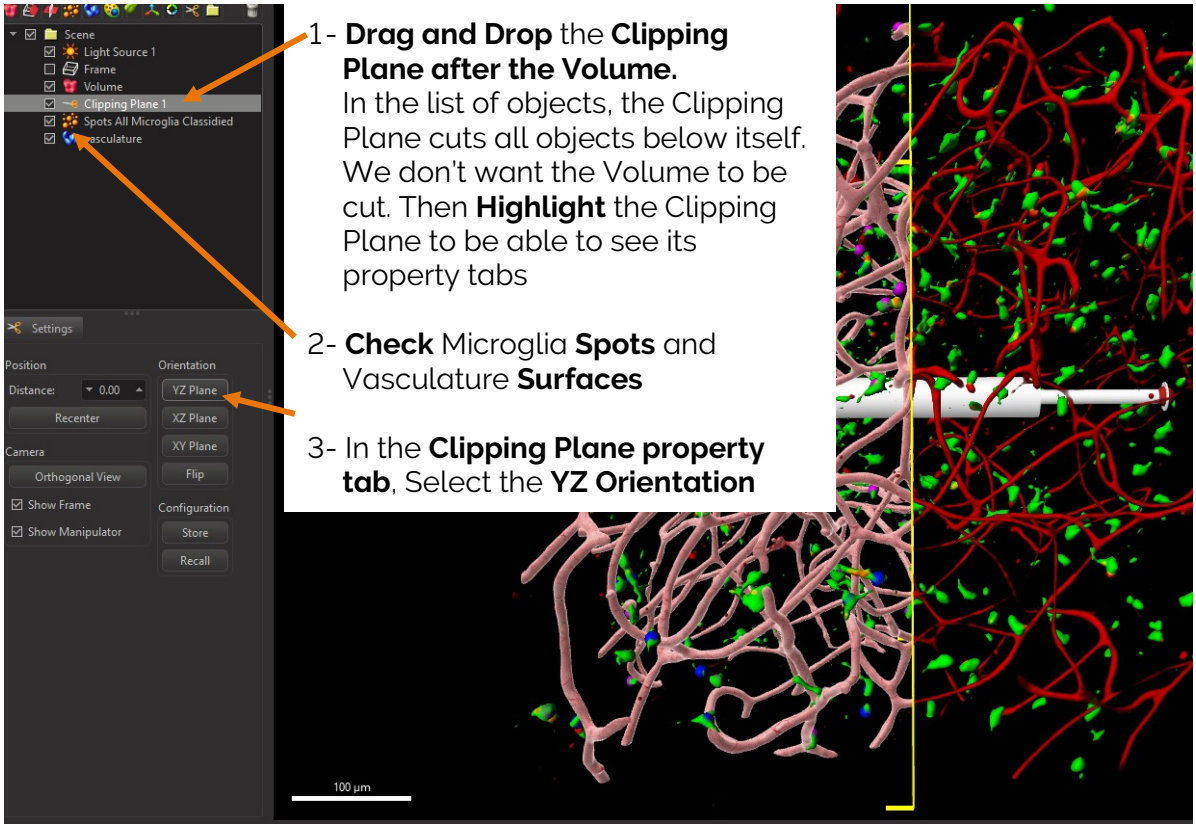
3- **Uncheck Microglia Spots** and **Vasculature Surfaces** so they are not displayed in the 3D Scene

4- In the **Volume Settings tab**, change the rendering mode to **Normal Shading** and **adjust** the transparency of each channel as shown

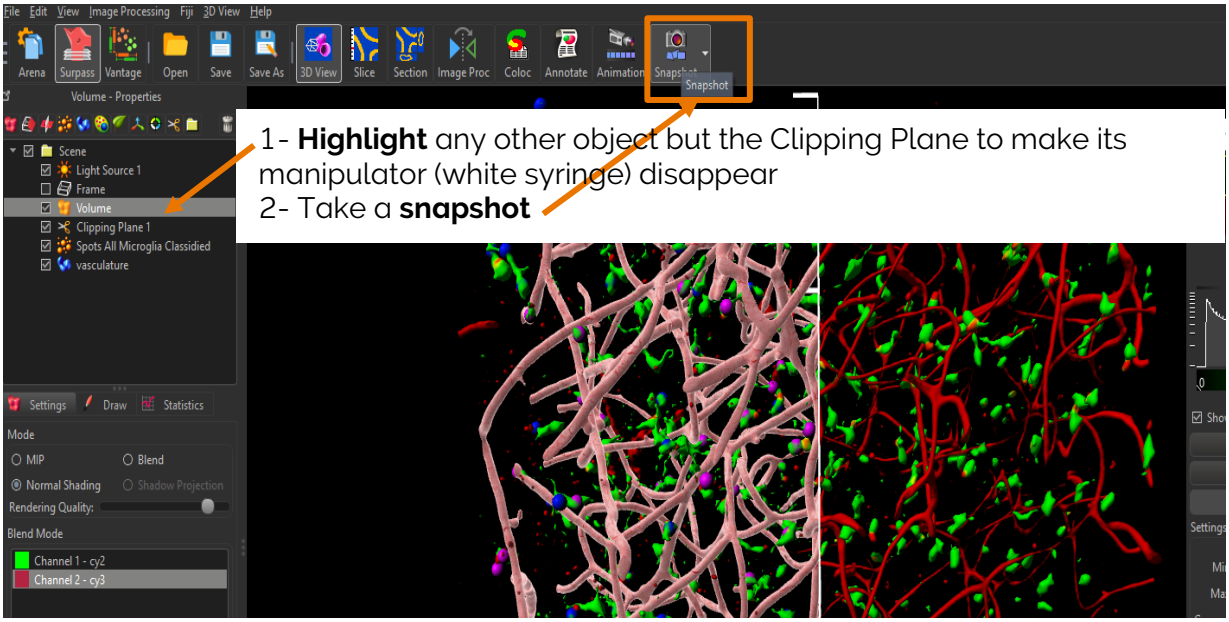



Add a Clipping Plane






Take a Snapshot




More for later…

**…about this image:**

You can explore the same dataset fully analysed for Microglia classification according to their activation state (ramification, shape, volume…): Dataset name **1-Complete-analysis-Microglia-Vasculature_Kofron**

**…about these topics:**

Imaris Spots detection algorithm
Blend and Normal Shading volume rendering modes tutorial
ShortestDistanceToSpots and ShortestDistanceToSurfaces

# Colocalization and intensity profile around nuclei

## Exercise 2 - 20 mins

### Dataset description

**File: 2-Nuclei_and_2_Prot_Coloc_Amita Gorur.ims**

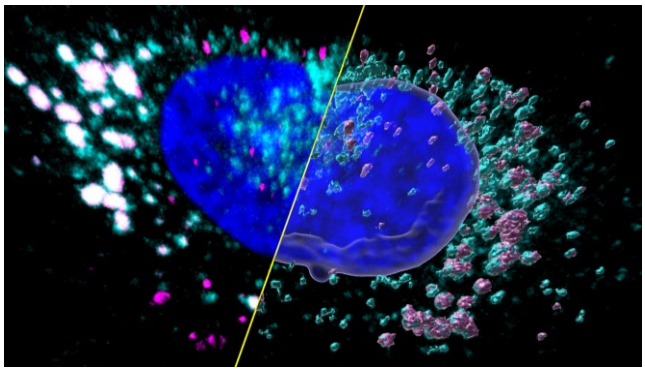
Channel 1 : Nuclei (DAPI) (Blue)
Channel 2 : Vesicles type 1 (Pink)
Channel 3 : Vesicles type 2 (Cyan)

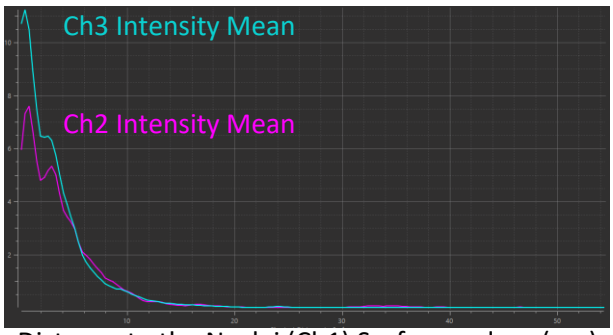
Courtesy of Dr. Amita Gorur and Dr. Randy Schekman, University of California,Berkeley, USA

### Scientific question and complete image analysis protocol

**Are specific vesicles more likely to be close to the nucleus membrane ? Do my two sets of vesicles colocalize and to which extent?**

1- Detect nuclei as Surfaces to get counts and volumetric and morphological statistics
2- Visualise the 2 vesicles types intensity channels spread around the nuclei Surfaces
3- Detect the 2 types of vesicles as Surfaces to get counts and volumetric and morphological statistics
4- Measure the overlapping regions between the 2 vesicle types Surfaces to evaluate their colocalization



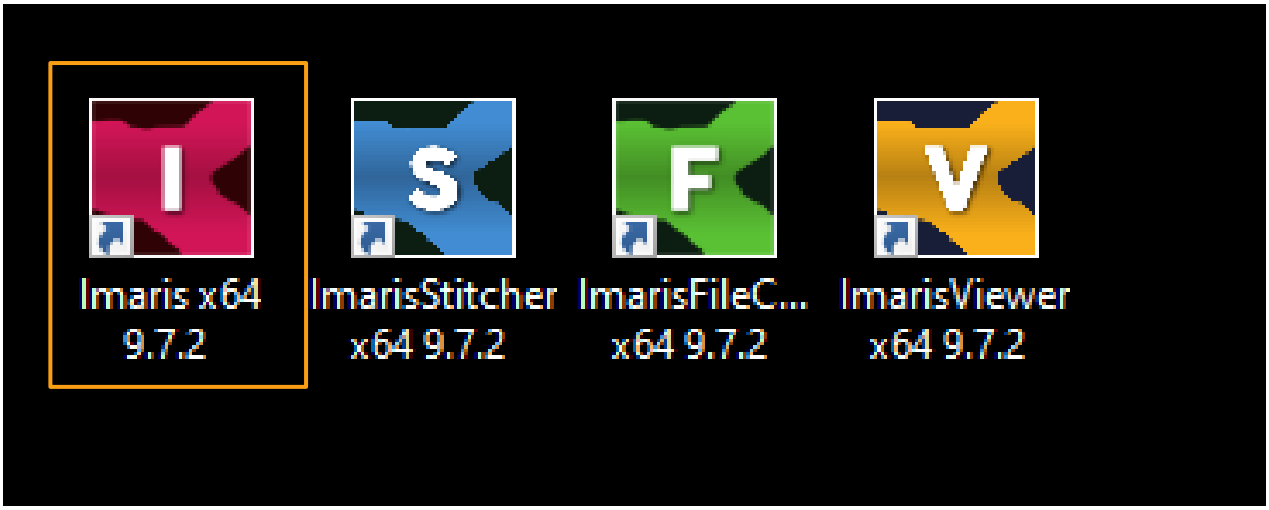


Distance to the Nuclei (Ch1) Surfaces edges (um)

### Step by step guided exercise

#### Open the image file:

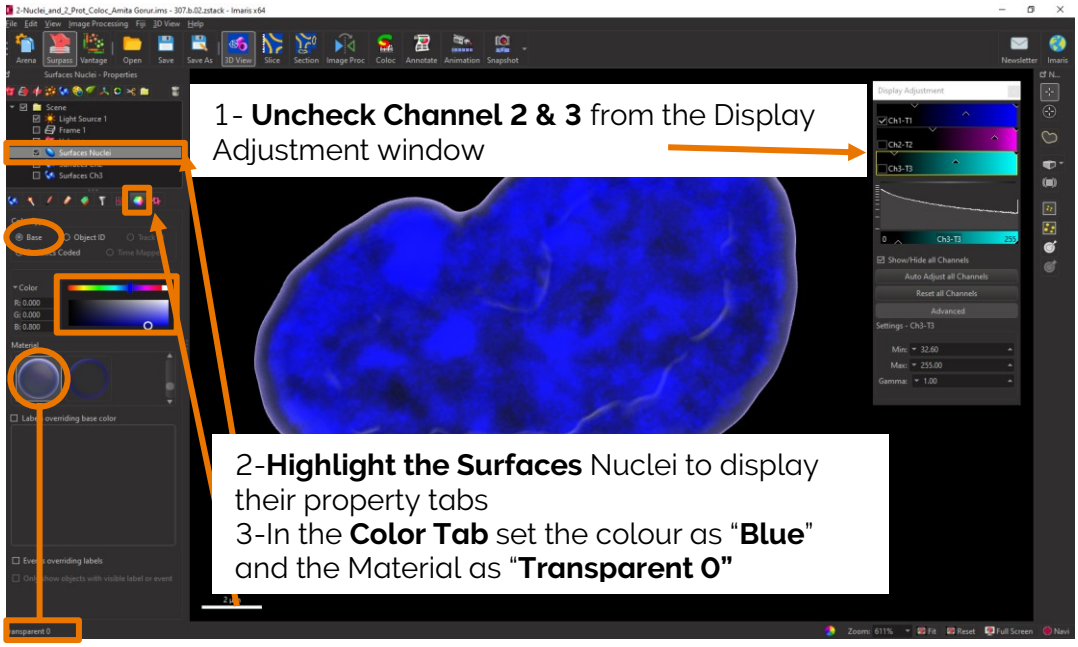
Open Imaris by double clicking on the main Imaris pink application icon:



Make sure you are in the "Arena" interface which is Imaris library of images and double click on the file **2-Nuclei_and_2_Prot_Coloc_Amita Gorur.ims**


## Surface Rendering Adjustment

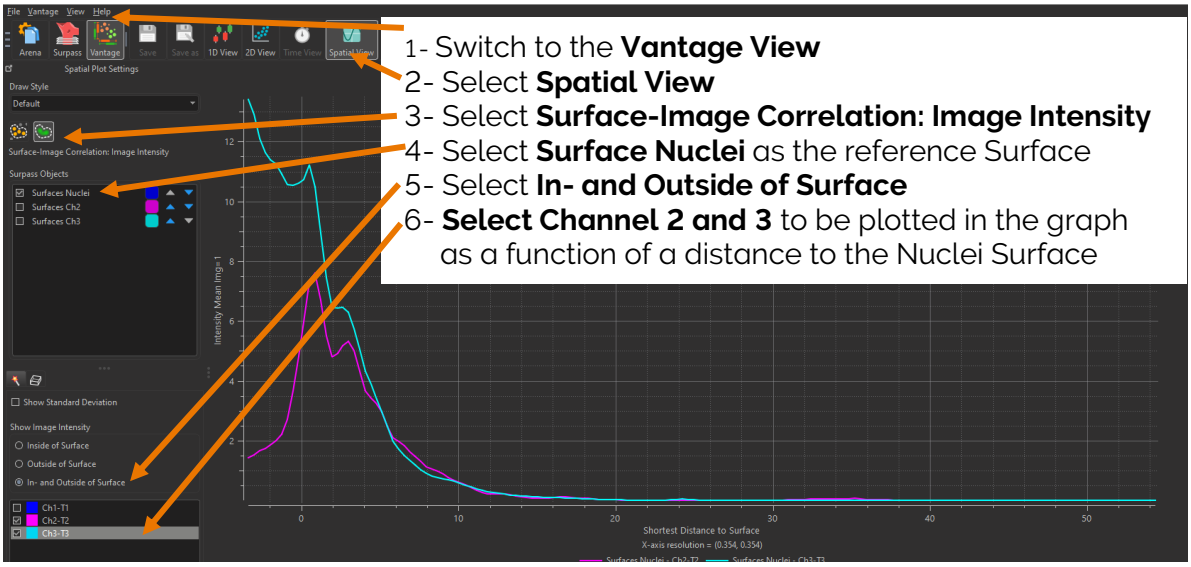
Adjust Nuclei Surfaces texture, transparency and colour to better visualize all vesicle types inside or outside the Nuclei..




Type Ctrl-D to open the Display Adjustment if it closed.
For more information on the Display Adjustment window Click this button

## Spatial View Surface Nuclei-Image Correlation: Image Intensity Plot

To verify if the two types of vesicles are more likely to be close to the nucleus membrane, we'll generate an intensity profile of their channels around the Nuclei.




Imaris Vantage here generates an intensity profile plot in all directions around the Nuclei Surfaces for each channel (Channel 2 & 3).

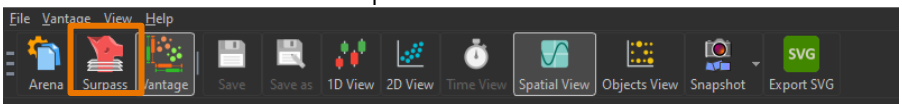
⇨ **We can see here, that most of the vesicles intensities are included in a shell of 4um around the Nuclei.**

The **Surface-Image Correlation: Image Intensity** allows for the comparison of the image intensity with respect to the Surface object.
The plot shows the average image intensity of each image channel versus distance d from the surface. This plot lets you inspect whether the intensities in some channel change as a function of distance from the surface. Clearly this is expected for the channel from which the surface was computed. Other channels may also exhibit a change of intensity with distance. Spatial statistics are calculated during surface object creation.

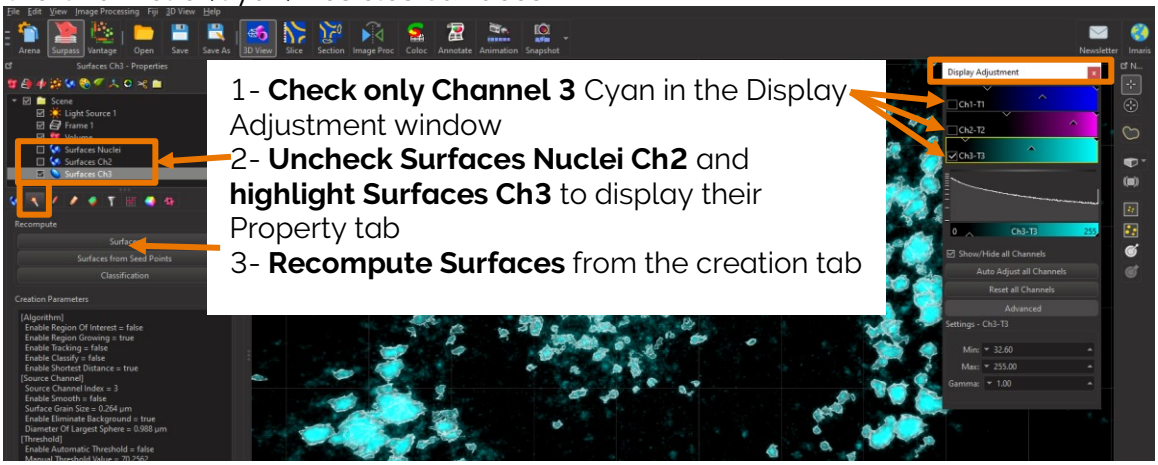
## Surface Creation Wizard: Rebuild

To check if the two sets of vesicles colocalize and if yes to which extend, we'll model each vesicle type as Surfaces and evaluate if there are any overlapping volumes.

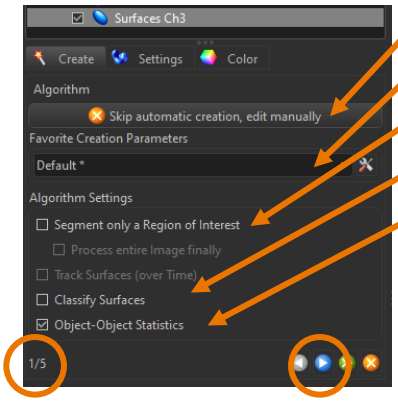
First switch back to the Surpass View :




Re-build the Channel 3 (Cyan) Vesicles Surfaces



## Surface Creation Wizard: Step 1/5




**Don't click,** we'll generate Surfaces automatically

**No parameters to load**

**Uncheck**

**Uncheck**

**Check,** we'll need the distance between the present Cyan vesicles Surfaces and the other type pink vesicles Surfaces

➢ Click on the **Blue arrow** circled to go to the next step

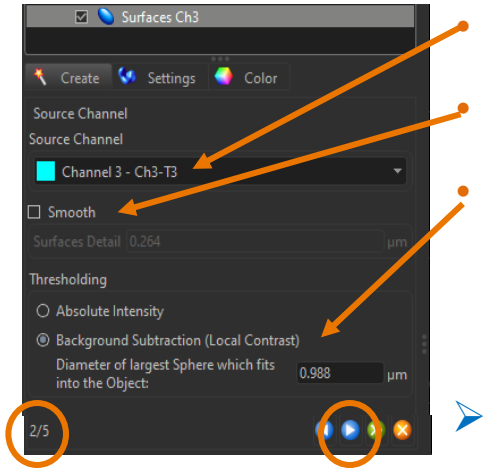
Option explanations:

- **Skip automatic creation, Edit manually**: if clicked, you'll skip all steps of the automatic creation wizard and you will have to create Surfaces manually.
- **Favourite creation parameters:** you can load previously saved parameters as favourite.
- **Segment only a Region Of Interest**: if checked, during the set up and optimisation process of the wizard of creation parameters, calculation will be restricted to an ROI so that it is fast. Once all steps of the wizard of creation are completed, calculation will run on to the full image. Here, we uncheck this box as we are working on a demo image however once you'll be working on your own dataset, best is to check it to gain in speed.
- **Classify Surfaces:** if checked, once Surfaces are detected, it enables automatic Surfaces Classification using either 1D or 2D filters or Machine Learning.
- **Object-Object statistics:** if checked, it enables the calculation of distances between objects.

➢ **Blue arrow:** to move from one step to the next one or previous one

➢ **Green arrow:** to finish the wizard of creation automatically using either default or loaded favourite or previously used parameters.

## Surface Creation Wizard: Step 2/5




Select **Ch3 Cyan** to quantify the vesicles type 2

**Uncheck** as here we prefer to have Surfaces detailed and not smoothed

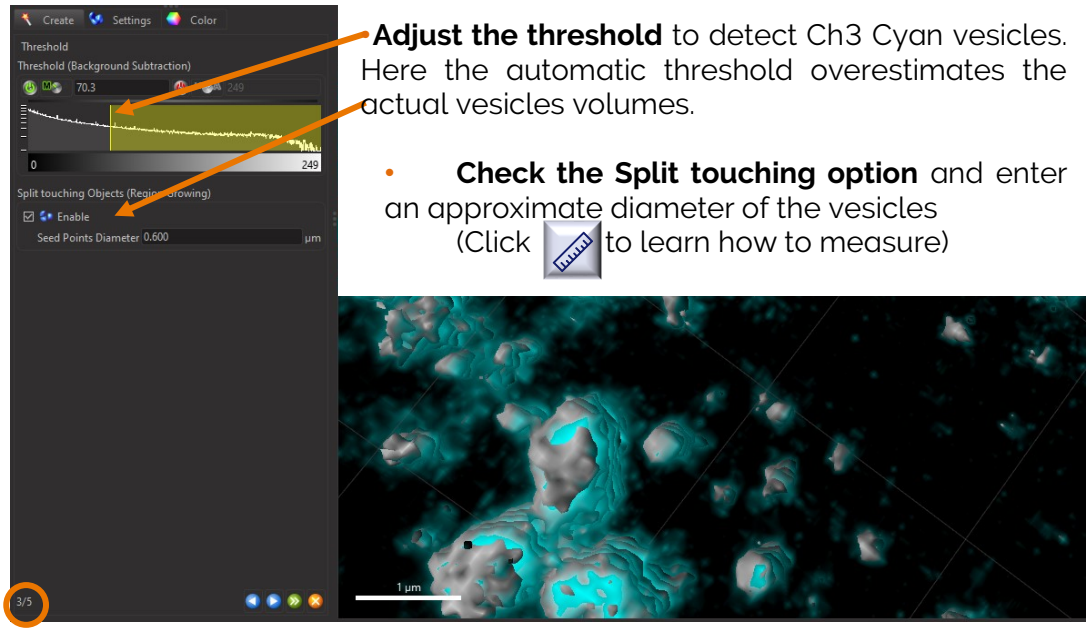
**Check** this box and use the default value.

➢ Click on the **Blue arrow** circled to go to the next step

Option explanations:

- **Smooth:** sets up the smoothness of the resulting area. Smoothing applies a Gaussian filter to the data set. If the option Smooth is selected, then the Surface Detailed Level value should be determined.
- **Background Subtraction:** if checked, it applies a Gaussian filter to estimate the background intensity value of each voxel. This variable background is then subtracted from every voxel in the image; Baseline Subtraction is performed. The width of the Gaussian filter is determined by the diameter of the largest sphere that fits into the Object from the intensity of the original channel.

## Surface Creation Wizard: Step 3/5



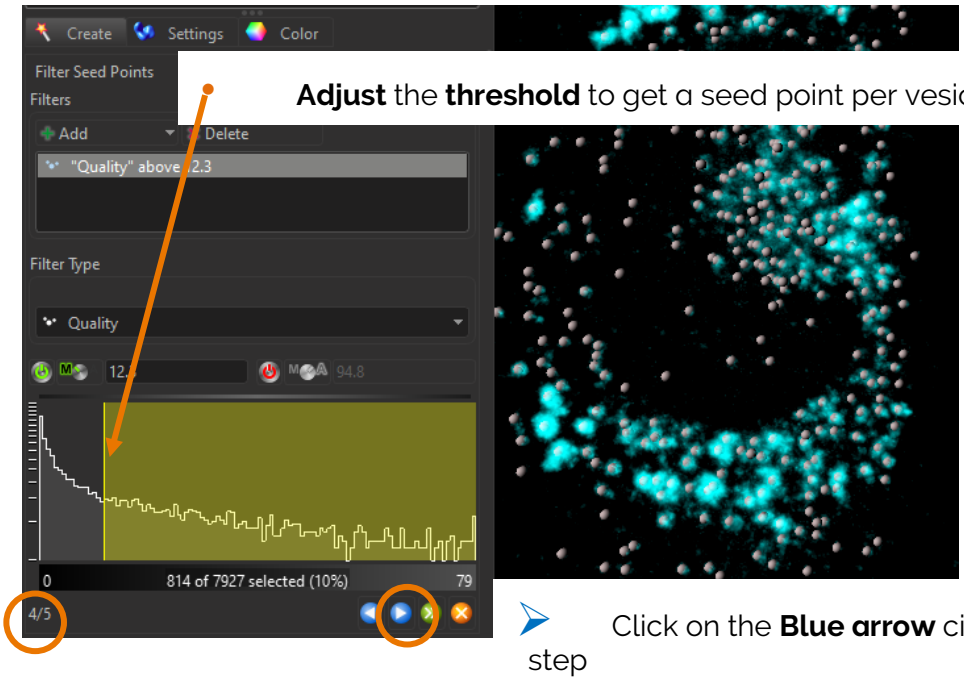

**Adjust the threshold** to detect Ch3 Cyan vesicles. Here the automatic threshold overestimates the actual vesicles volumes.

- **Check the Split touching option** and enter an approximate diameter of the vesicles (Click  to learn how to measure)

Option explanations:

- **Threshold**: Objects with values below the Lower Threshold and objects with values exceeding the Higher Threshold will be excluded and only those objects with quality inside the interval defined by the two threshold values are considered.
- **The Green A stands for Automatic thresholding**: the initial threshold value is automatically computed using an algorithm based on [T.W. Ridler and S. Calvard, "Picture thresholding using an iterative selection method", IEEE Trans. System, Man and Cybernetics, SMC-8 (1978) 630-632]. If you do a Manual thresholding, then you'll see a green M
- **Split touching Objects (Region Growing):** This option supports the separation of two or more objects that are identified as one. The region method for segmentation in Imaris starts with defining seed points in each peak center. Then a region growing process will run around each seed point where the region grows until a border stops the growing. The threshold you set above is the border of the region growing

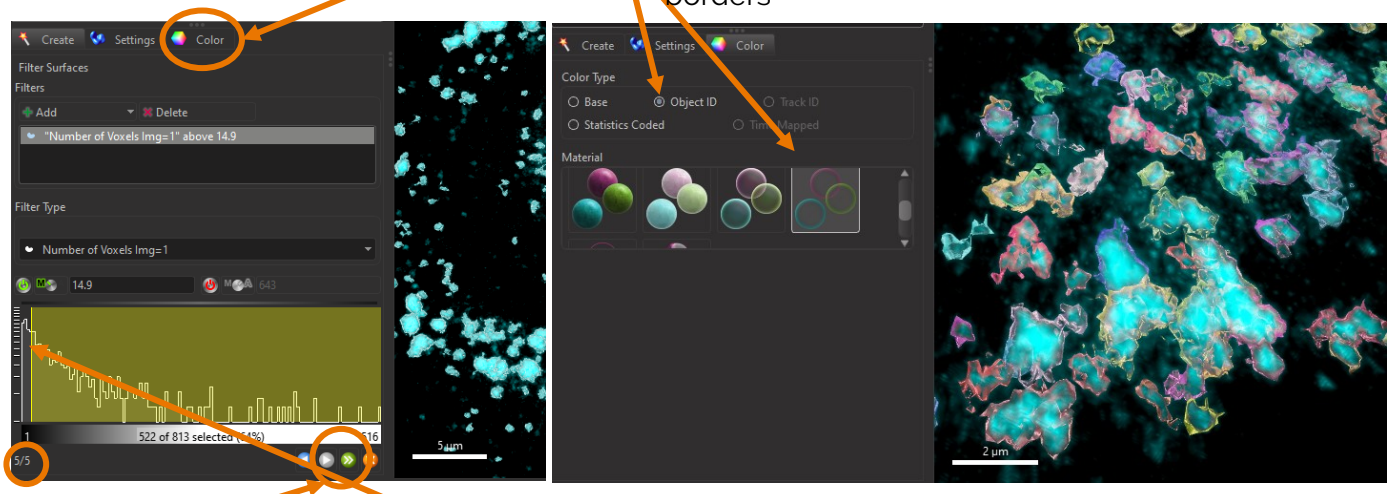
## Surface Creation Wizard: Step 4/5




**Adjust** the **threshold** to get a seed point per vesicle

Click on the **Blue arrow** circled to go to the next step

1. **Click** on the **Color tab**
2. Give each Surface a **random colour** and **transparency** suitable for visualization of Surfaces borders



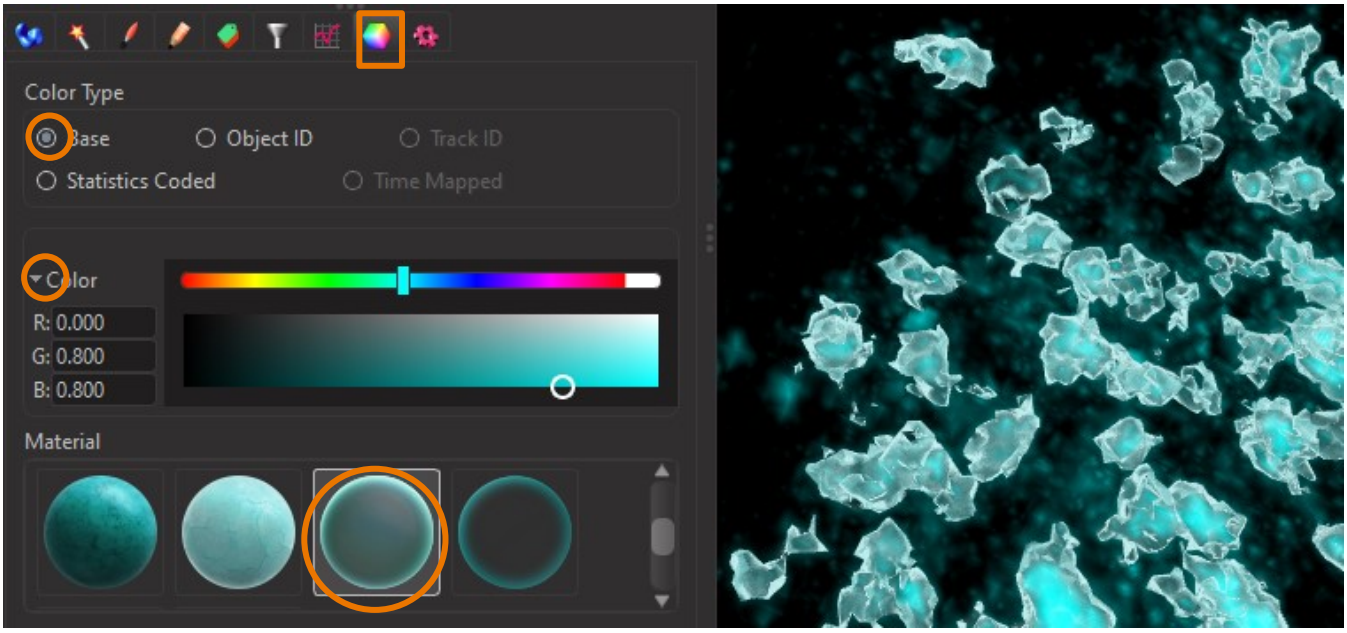

3. **Inspect the Surfaces (vesicles) splitting**
4. **Adjust** the **threshold** to eliminate small Vesicles Surfaces

➢Click on **the green arrow** to finish the wizard of creation and get the Surfaces property tabs

## Surfaces Color tab

The Surfaces wizard of creation turns into a list of Surfaces property tabs (Click  to explore properties)

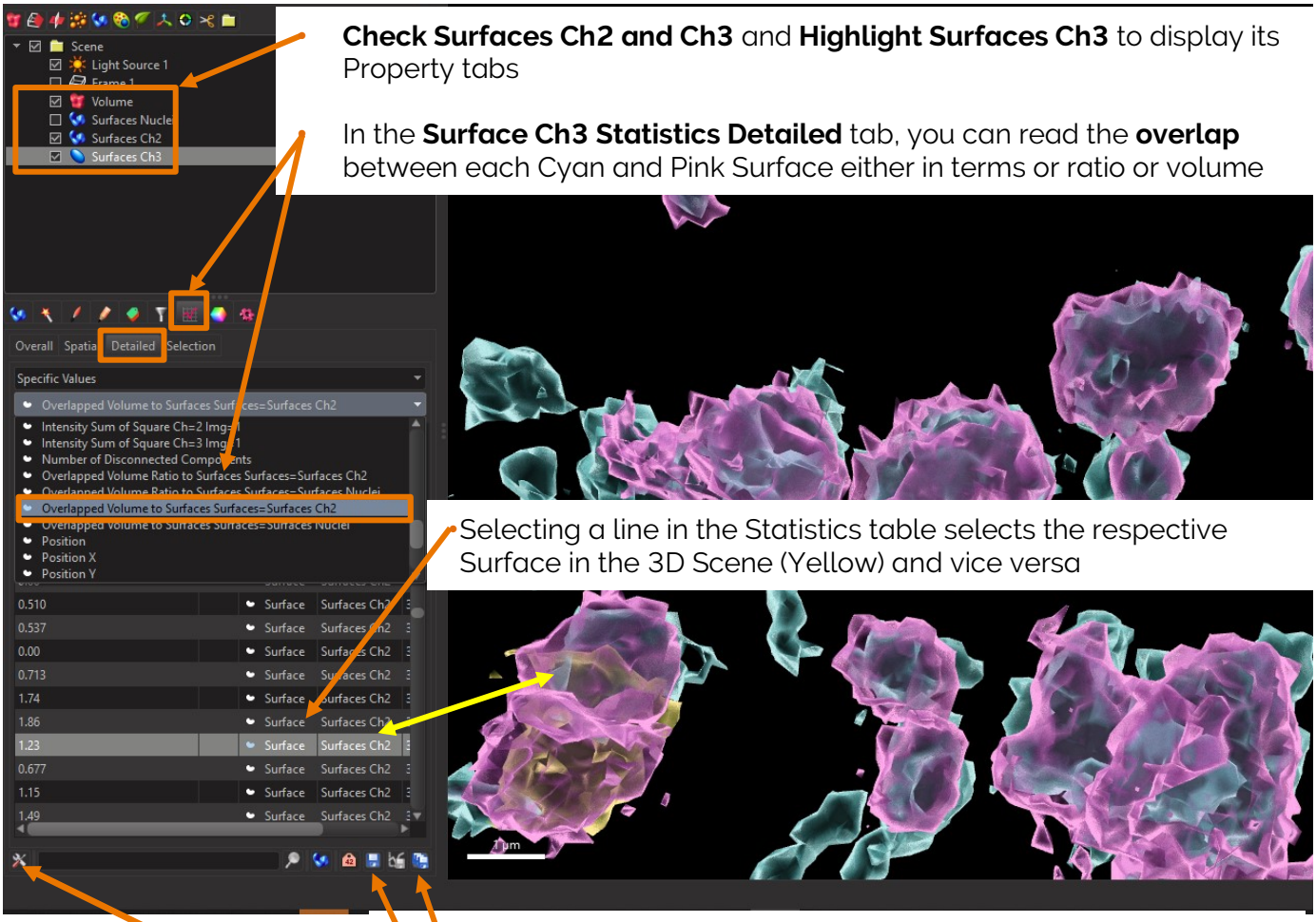
In the **Color tab**, select **Base** then the **Cyan** colour and the **Transparent 0 material**



## Surfaces Statistics

In this exercise, we are interested into measuring the colocalization between the two types of vesicles. One way of measuring colocalization is to measure the overlapping between the Pink and and Cyan Surfaces. The statistics are called : :

a. Overlapped Volume to Surfaces Surfaces
b. Overlapped Volume Ratio to Surfaces




**Check Surfaces Ch2 and Ch3** and **Highlight Surfaces Ch3** to display its Property tabs

In the **Surface Ch3 Statistics Detailed** tab, you can read the **overlap** between each Cyan and Pink Surface either in terms or ratio or volume

Selecting a line in the Statistics table selects the respective Surface in the 3D Scene (Yellow) and vice versa


**Exports all** calculated **statistics** as preformatted .xls, .csv or xml file

**Exports** only the **observed statistic** in the table

**Opens** the **Preferences window** under the Surfaces Statistics where you can select or deselect any Statistic

## More for later…

Imaris Surfaces Detection algorithm
Colocalization tutorial and webinar

# Cell density and classification per region

## Exercise 3 - 15 mins

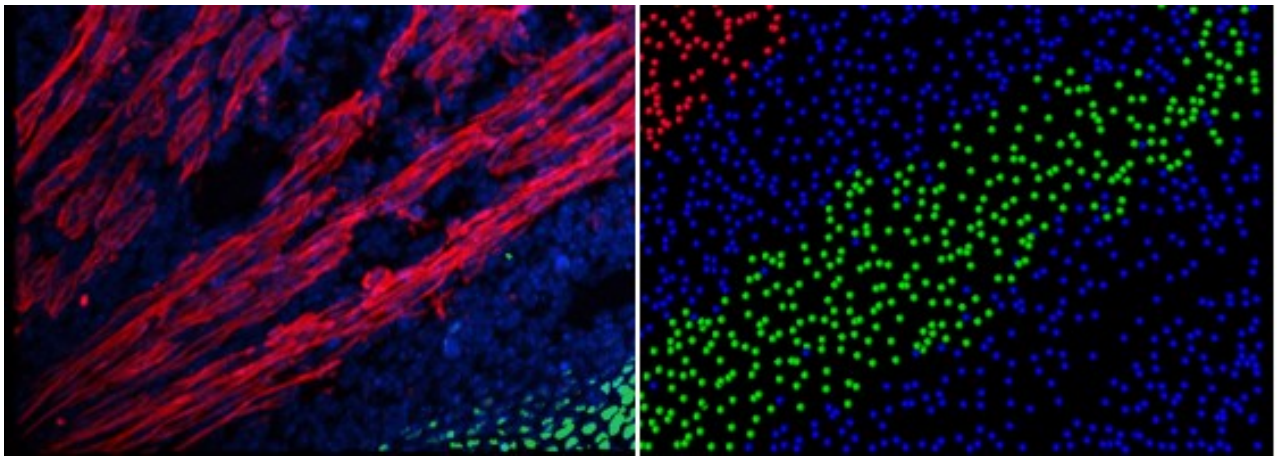
### Dataset description

**File: 3-Plant_Cells_ML_Clasification.ims**
Channel 1 : Nuclei (DAPI) (Blue)
Channel 2 : GFP (Green)
Channel 3 : RFP (Red)

Plant Cells image collected at the Institute Pasteur Paris with an Andor DragonFly spinning disk, Courtesy of Dr. Bruno Combettes, Andor Technology Ltd., Belfast, UK

### Scientific question and complete image analysis protocol

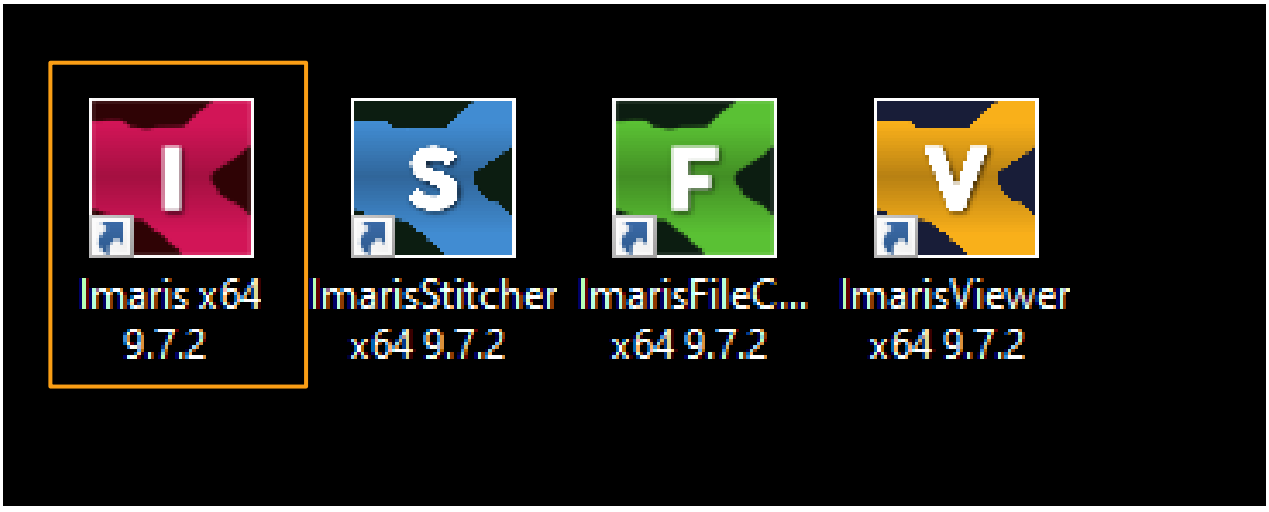
**Measure cell density over different regions of interest that are not defined by a specific staining?**
1- Detect Nuclei as Spots to get their count and positions
2- Classify Nuclei per Region Of Interest using Machine Learning
3- Evaluate densities of each subpopulation and compare them



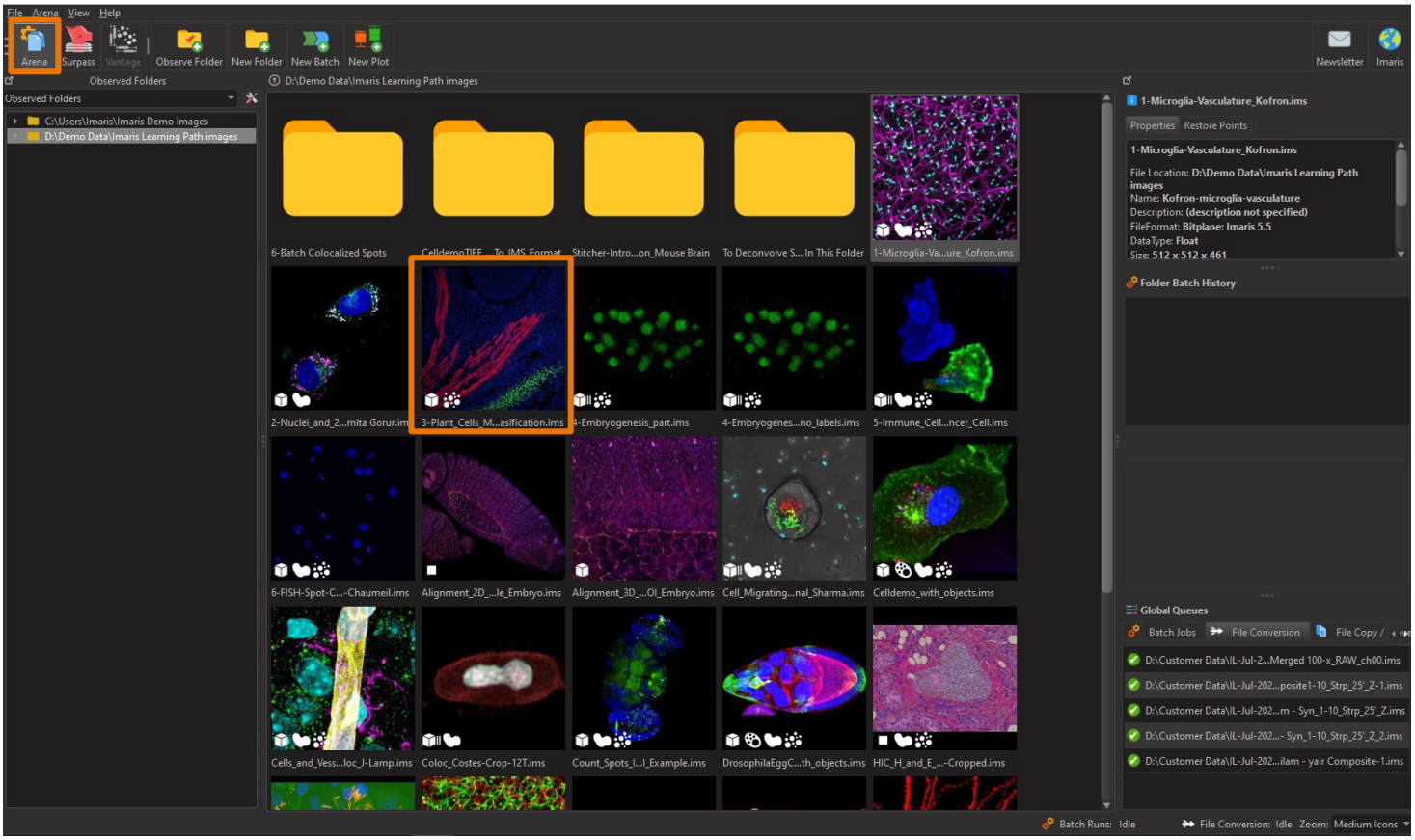
### Step by step guided exercise

#### Open the image file:

Open Imaris by double clicking on the main Imaris pink application icon:

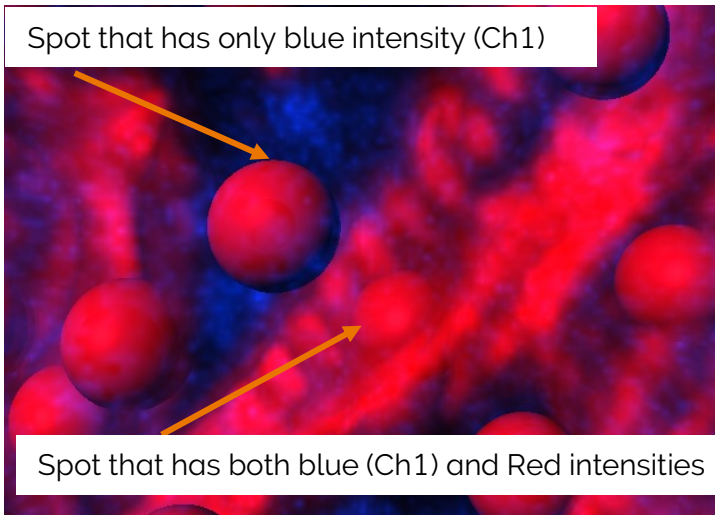


Make sure you are in the “Arena” interface which is Imaris library of images and double click on the file **3-Plant_Cells_ML_Clasification.ims**

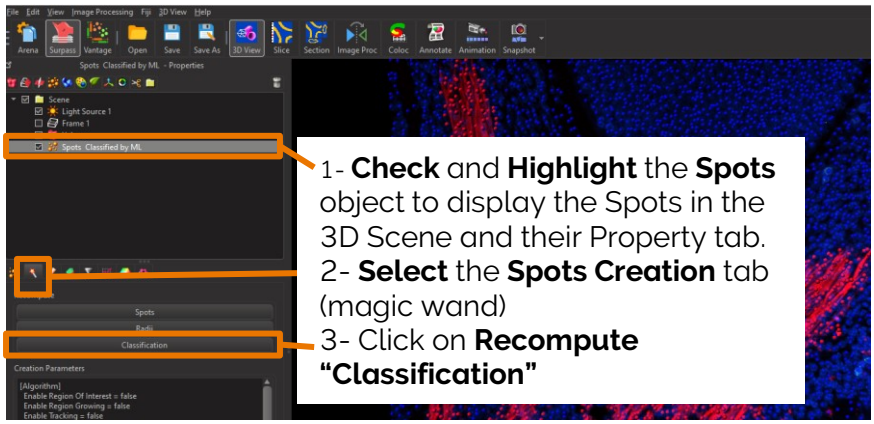



## Spots Machine learning (ML) Classification: Rebuild

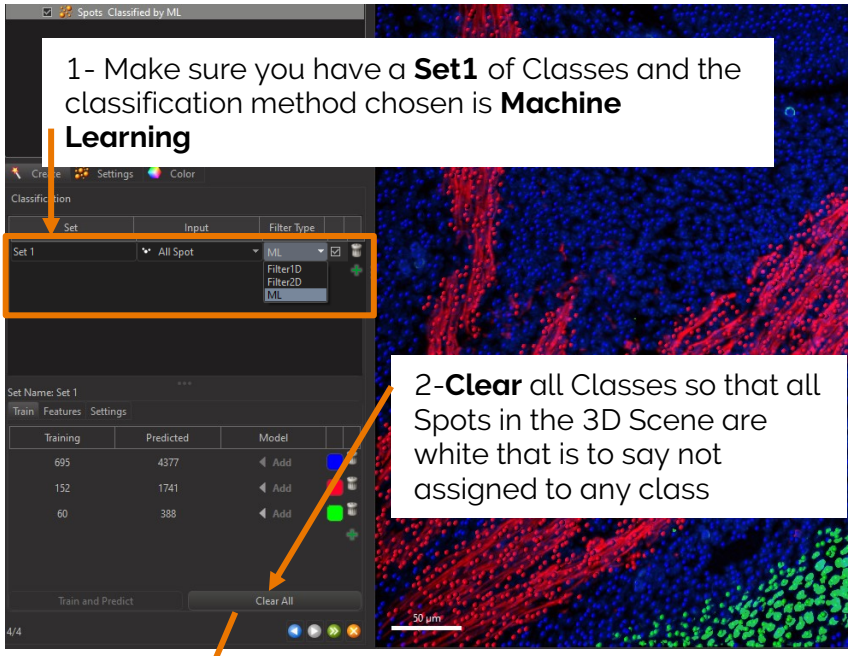
We will classify Nuclei per region using Machine Learning. Here, the difficulty of the classification lies in the fact that a colocalization study would not describe these 3 regions correctly. For instance, some Spots should be classified in the Red region whereas their blue DAPI intensity does not colocalize with the Red Channel. Below, a zoom in the “Red” region where Spots are classified as “Red” Class:.

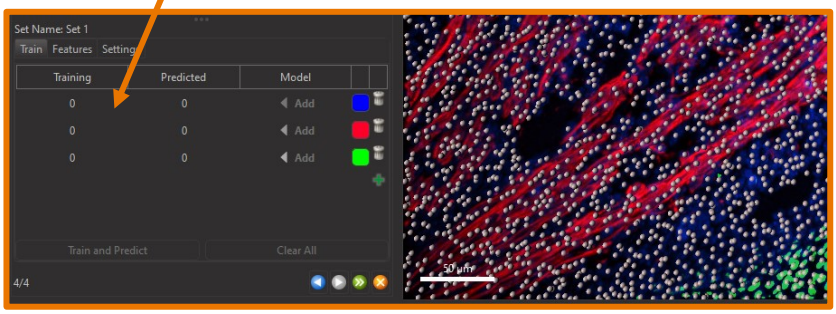


Let's rebuild the existing classification. The very first step could take a minute or 2 as Imaris is re-calculating all Machine Learning specific Statistics. (click  to explore Machine Learning statistics)

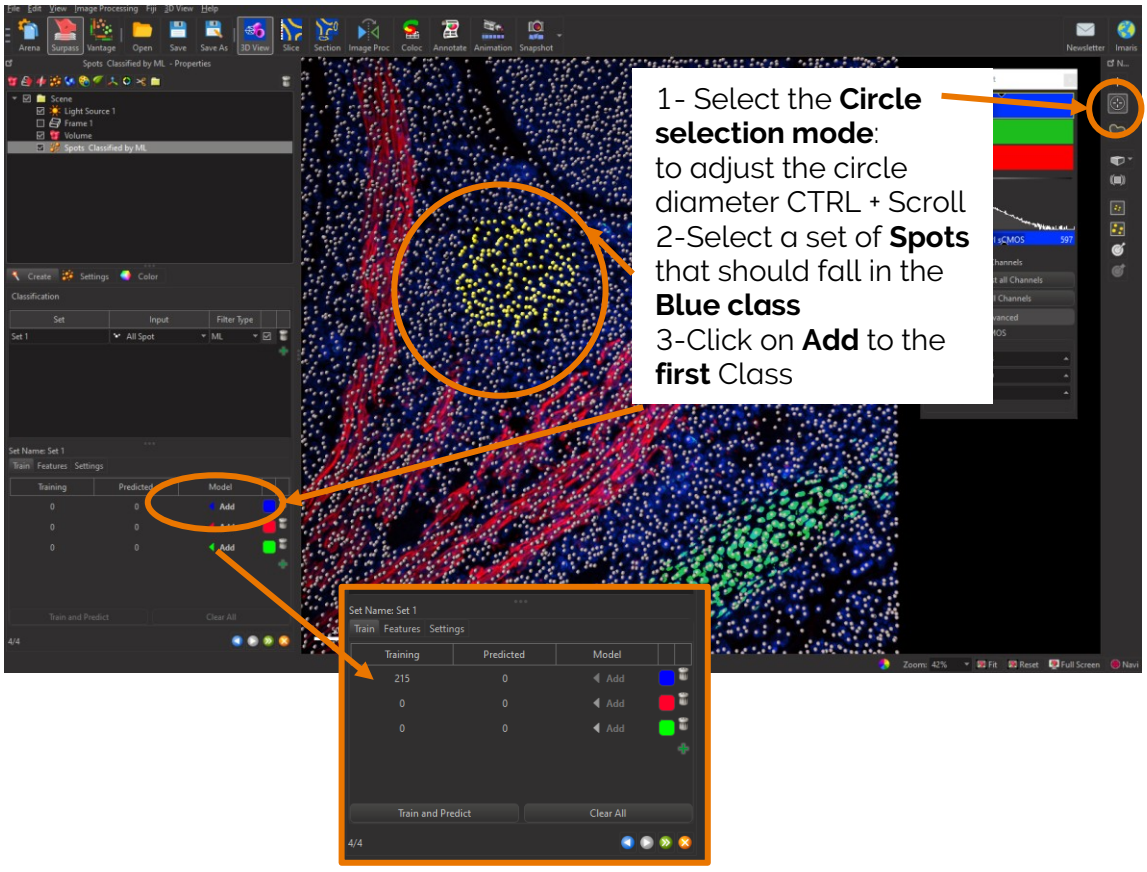



## Spots Machine learning Classification: Clear Training and Predicted sets

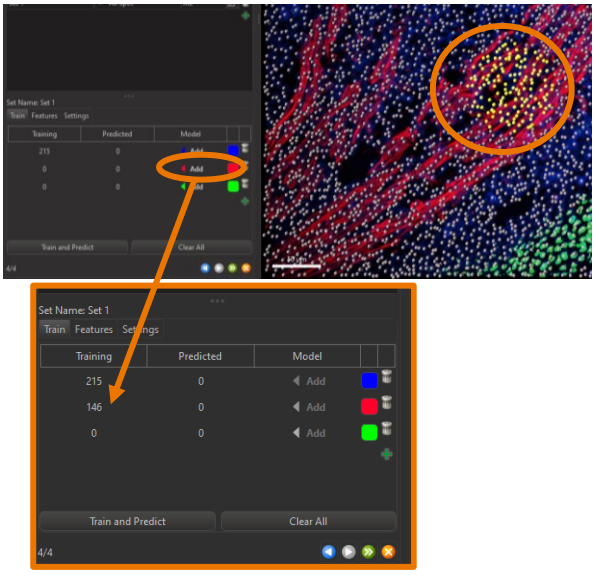


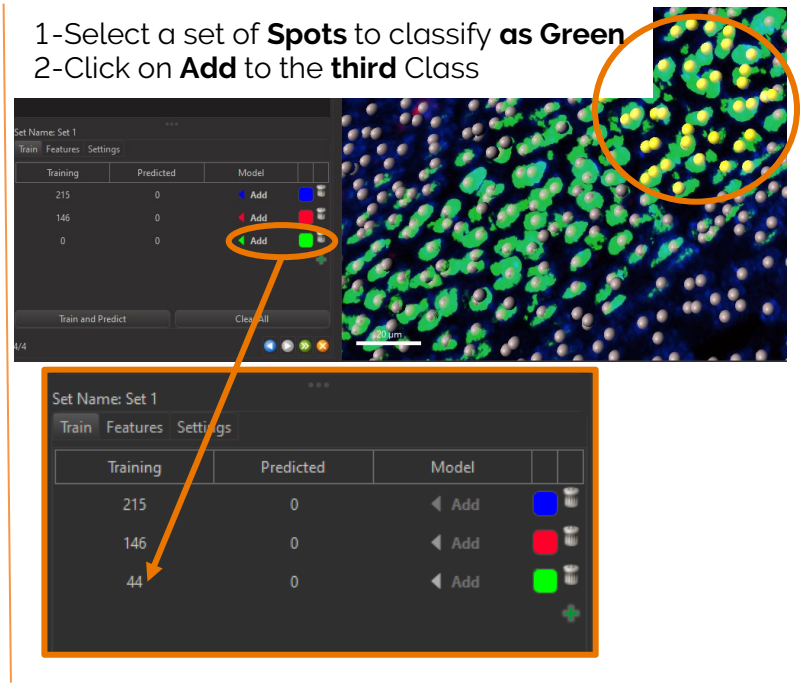
## Spots Machine learning Classification: Select and train




1-Select a set of **Spots** to classify as **Red**
2-Click on **Add** to the **second** Class

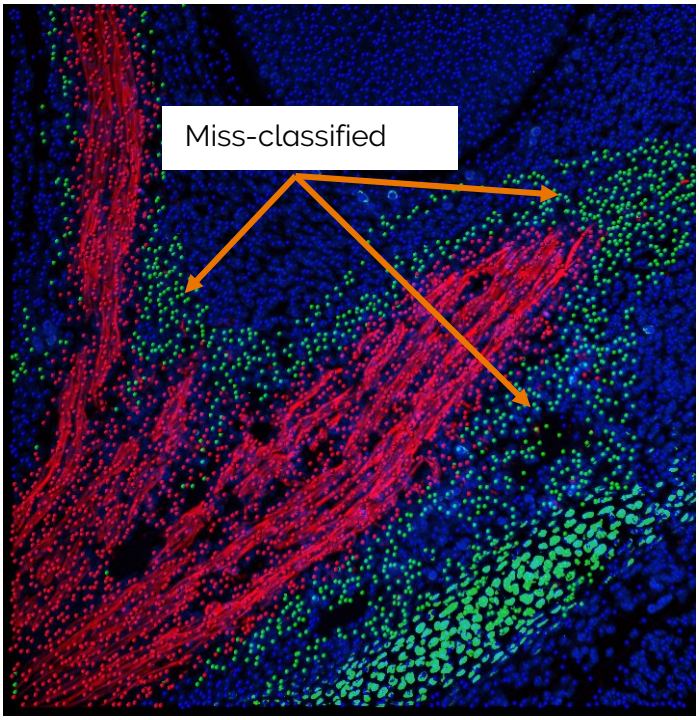



1-Select a set of **Spots** to classify **as Green**
2-Click on **Add** to the **third** Class

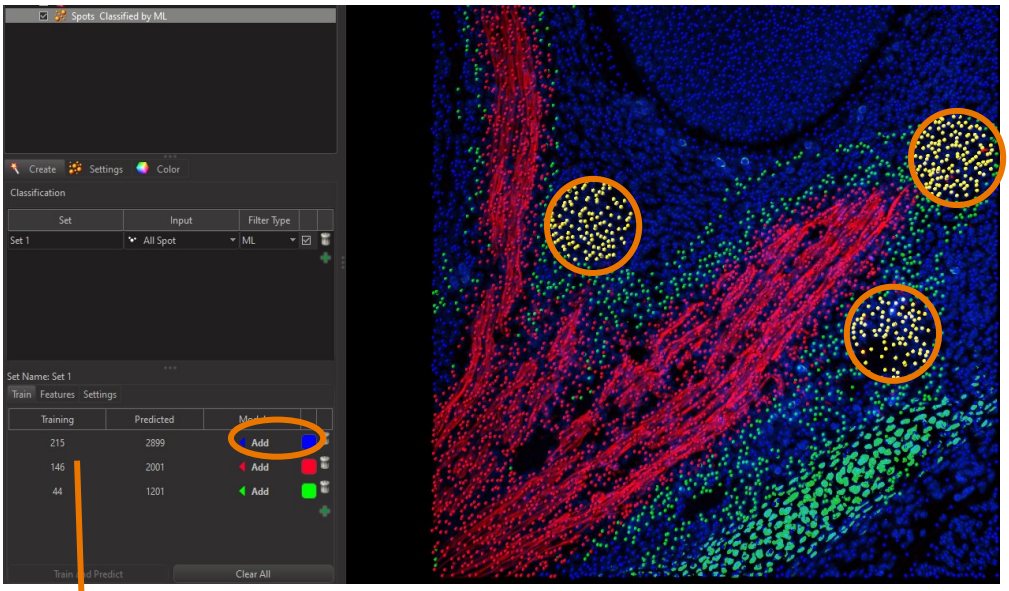


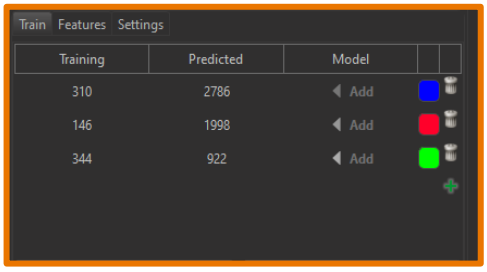

Train your model by Clicking Train and Predict

Review the results



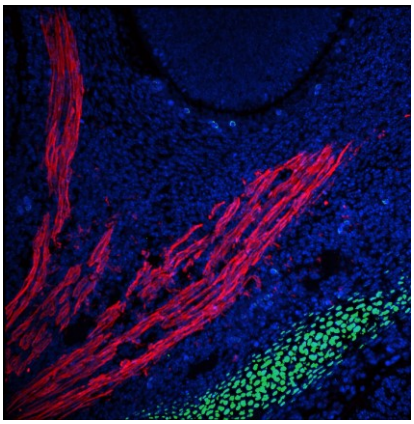

**Correct mistakes**: here, we **select** some Spots **miss-classified** in the third class green and **Add** them to the **correct Class** first class Blue. To select multiple circular regions, hold CTRL and click.

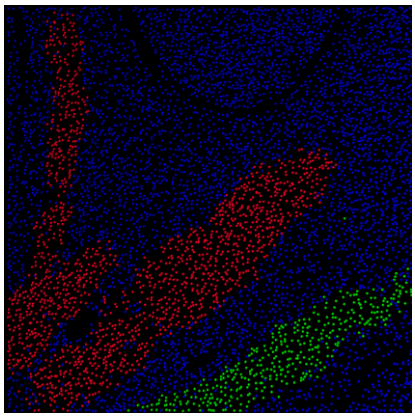


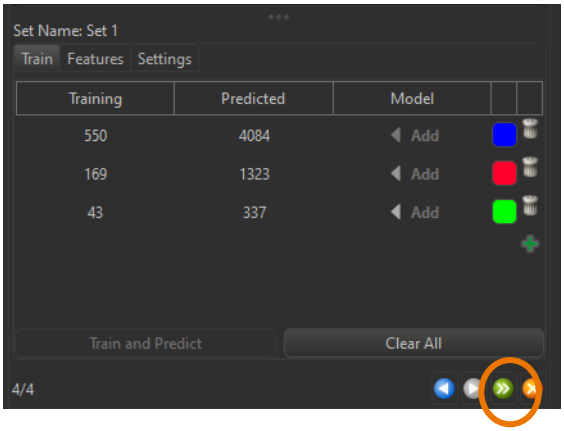



**Re-train** your model again by clicking again on **Train and Predict**


Re-iterate manual corrections and "Train and Predict" until all Spots are correctly classified and then click on the green arrow to finish the classification



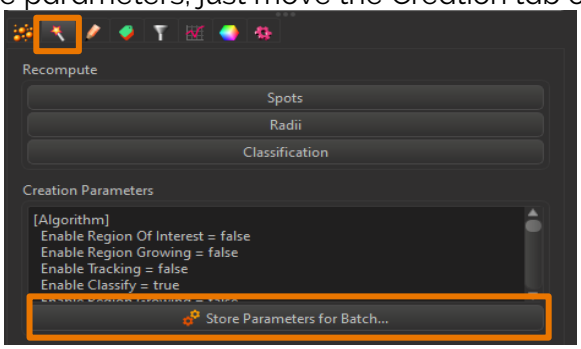




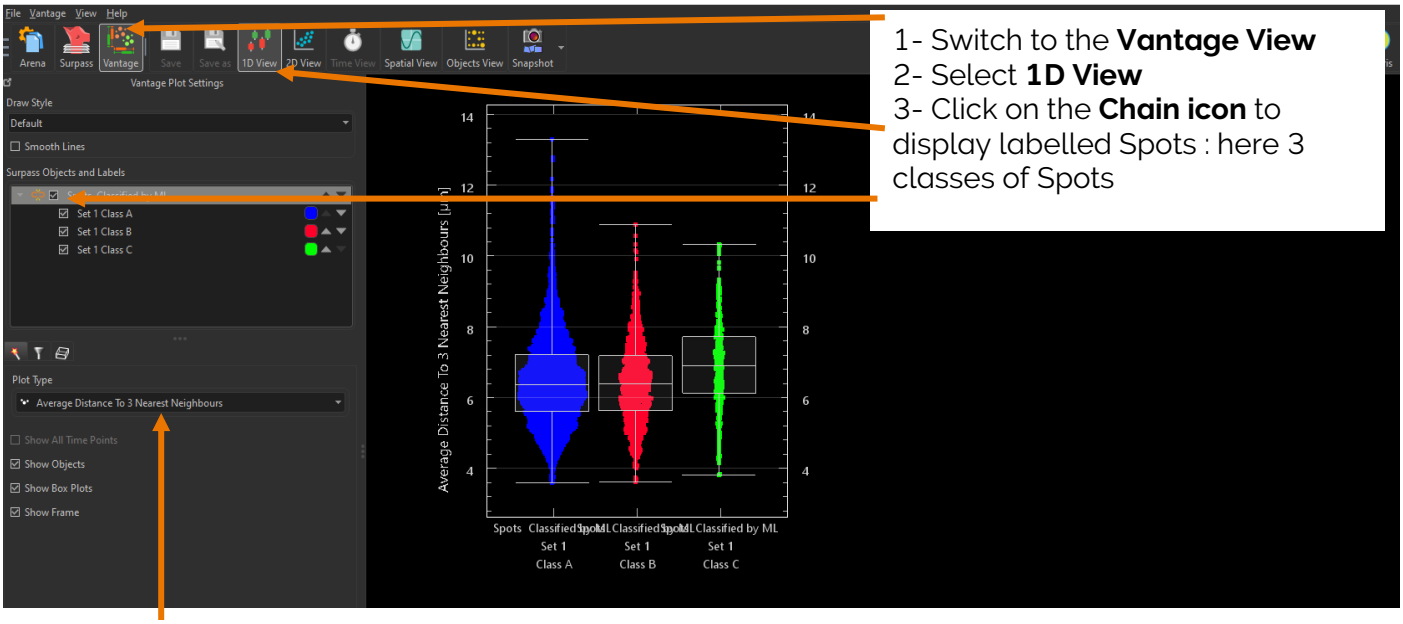

⇨ **Finally, all Nuclei Spots were correctly classified by the Machine Learning method.**

This method takes advantage of all standard statistics calculated by Imaris (including Average Distance to Nearest Neighbour) and additional "Machine Learning Statistics", which provide more information about local intensity landscape, "texture", intensity on the edges and shape information.

The trained model can be saved within all creation parameters as favourite parameters and for Batch processing.
You can re-used the saved parameters and train even more your model on other images and then saved the improved model.
To save parameters, just move the Creation tab of the created objects and save.



## 1D comparison Plot: Classes Densities




1- Switch to the **Vantage View**
2- Select **1D View**
3- Click on the **Chain icon** to display labelled Spots : here 3 classes of Spots

4- Test a few density statistics such as :

- **SpotsAverageDistanceTo3NearestNeighbours**

Measures the average distance to the nearest 3 spots within the same spots component.

- **SpotsAverageDistanceTo5NearestNeighbours**

Measures the average distance to the nearest 5 spots within the same spots component.

- **SpotsAverageDistanceTo9NearestNeighbours**

Measures the average distance to the nearest 9 spots within the same spots component.

⇨ **The SpotsAverageDistanceToNearestNeighbours statistics provides useful density measurements for the spots. Where the average distance is large, density is low. These statistics can be useful for discriminating regions with different spot densities.**

More for later : Classification concept and Machine Learning

# Changes in cell speed and intensity over time

## Exercise 4 - 30 mins

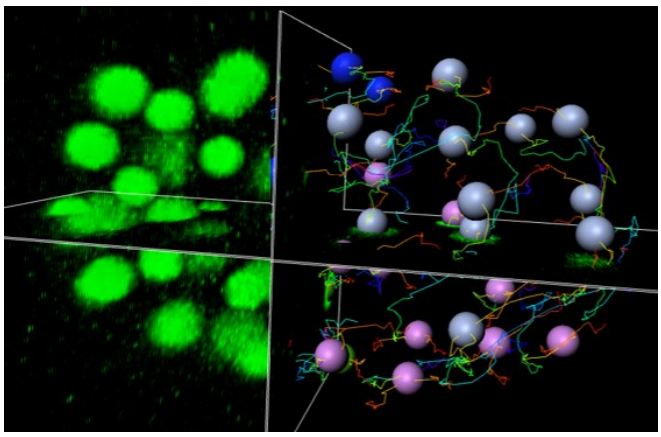
### Dataset description

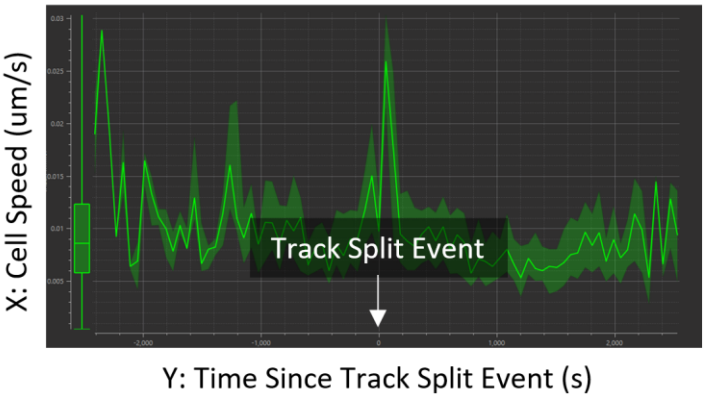
**File: 4-Embryogenesis_part_no_labels.ims**
Channel 1 : GFP -Histone (Green)
Drosophila development early stage

Courtesy of Dr. Matthew Kofron, Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA

### Scientific question and complete image analysis protocol

**How cell dynamics and intensities vary over the cell cycle?**
1- Detect and track cells over time as Spots
2- Label cells (Spots) per developing embryo region
3- Analyse Cell behaviours before and after they Split

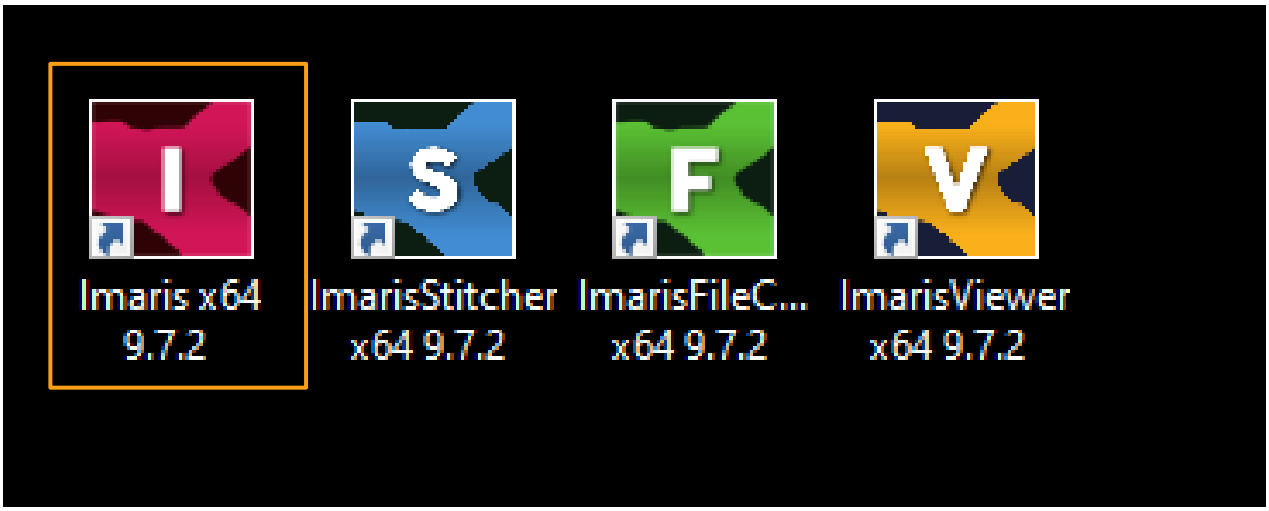





### Step by step guided exercise

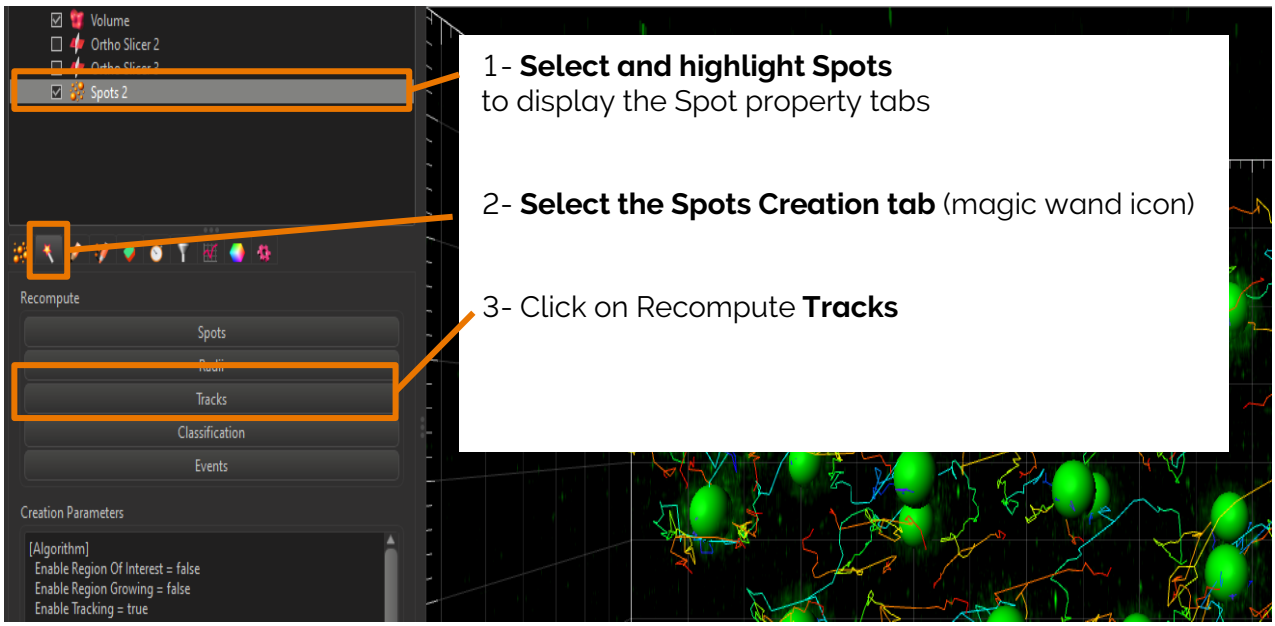
#### Open the image file:

Open Imaris by double clicking on the main Imaris pink application icon:

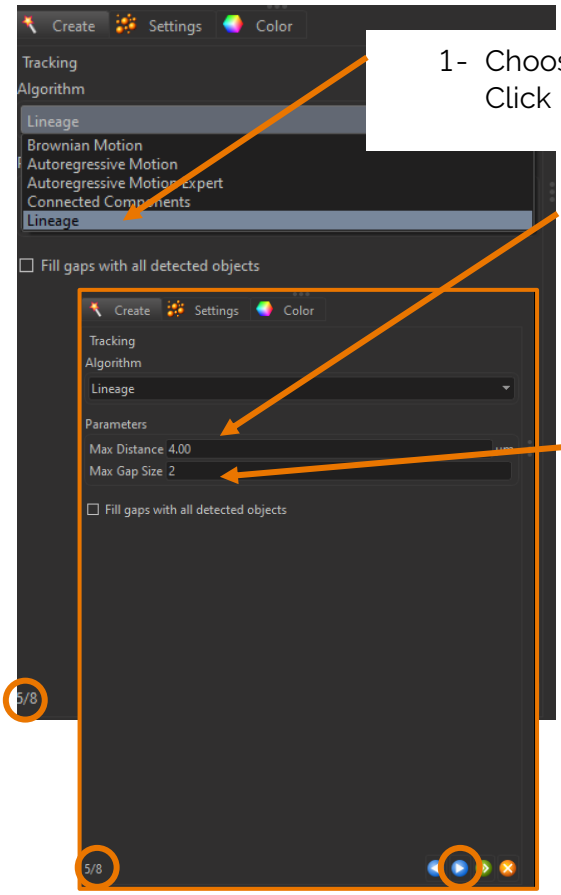


Make sure you are in the "Arena" interface which is Imaris library of images and double click on the file **4-Embryogenesis_part_no_labels.ims**


## Spots Tracking: Rebuild



## Spots Tracking: Algorithm



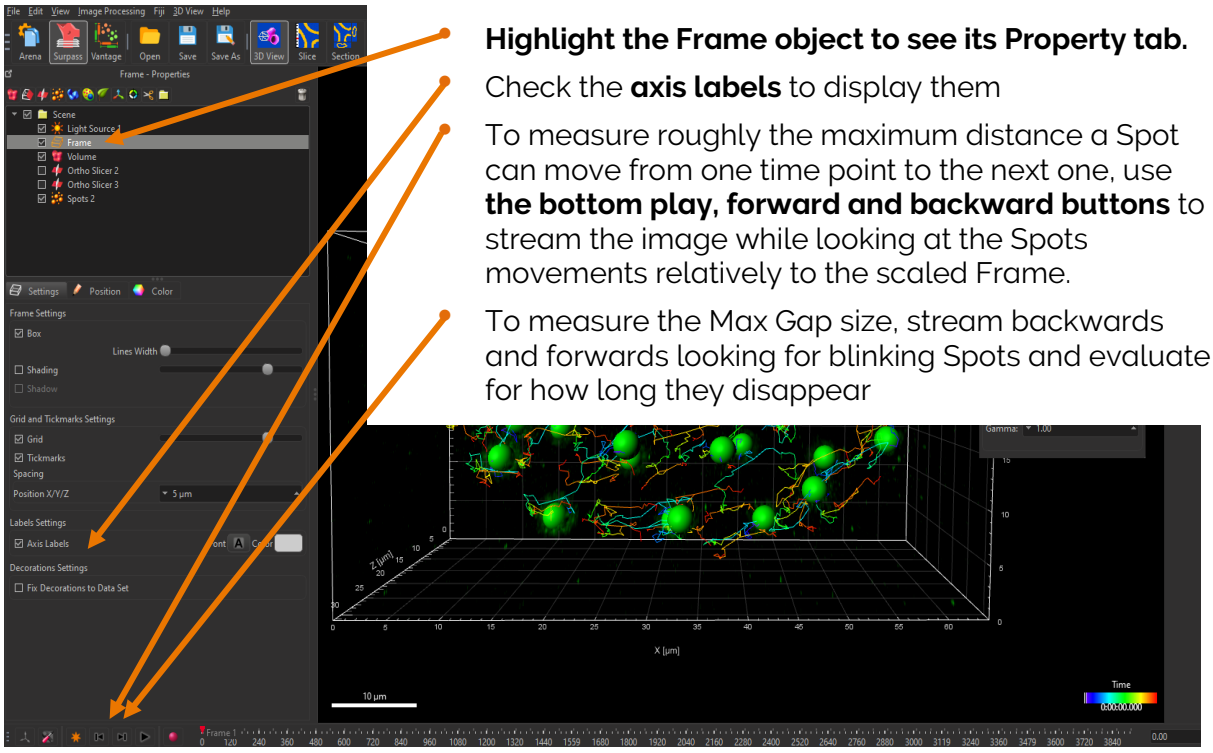

1- Choose **Lineage algorithm** from the drop-down menu
Click (i) to explore all tracking algorithms)

2- Set the **Max Distance** to **4um**
This is the maximum distance a Spot can move from one time point to the next one.
To roughly measure it, see below

3- **Set the Max Gap size to 2**
This defines the maximum number of the consecutive time points where a Spot is allowed to be missing in order for its track to continue and not break.
To roughly measure it, see below

➢ Click on the **Blue arrow** circled to move to the next step

## Spots Tracking: Measure Max Distance and Gap Size:



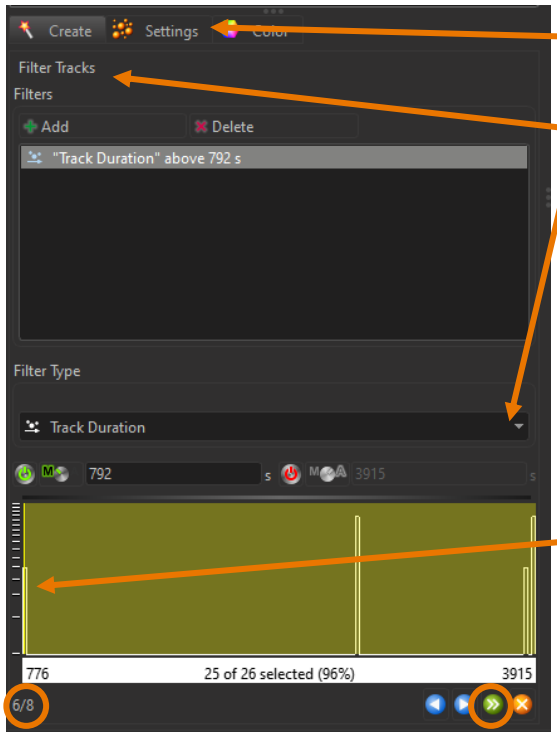

**Highlight the Frame object to see its Property tab.**

Check the **axis labels** to display them

To measure roughly the maximum distance a Spot can move from one time point to the next one, use **the bottom play, forward and backward buttons** to stream the image while looking at the Spots movements relatively to the scaled Frame.

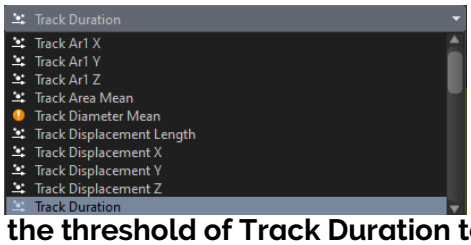
To measure the Max Gap size, stream backwards and forwards looking for blinking Spots and evaluate for how long they disappear

## Spots Tracking: Filter Tracks




! Check in the Spots Settings tab that Spots are displayed as spheres

This Filter Tracks step allows the removal of short tracks that could be noise, artefacts or any other unwanted structures tracked. Any calculated statistic can be chosen from the drop-down menu to filter out tracks



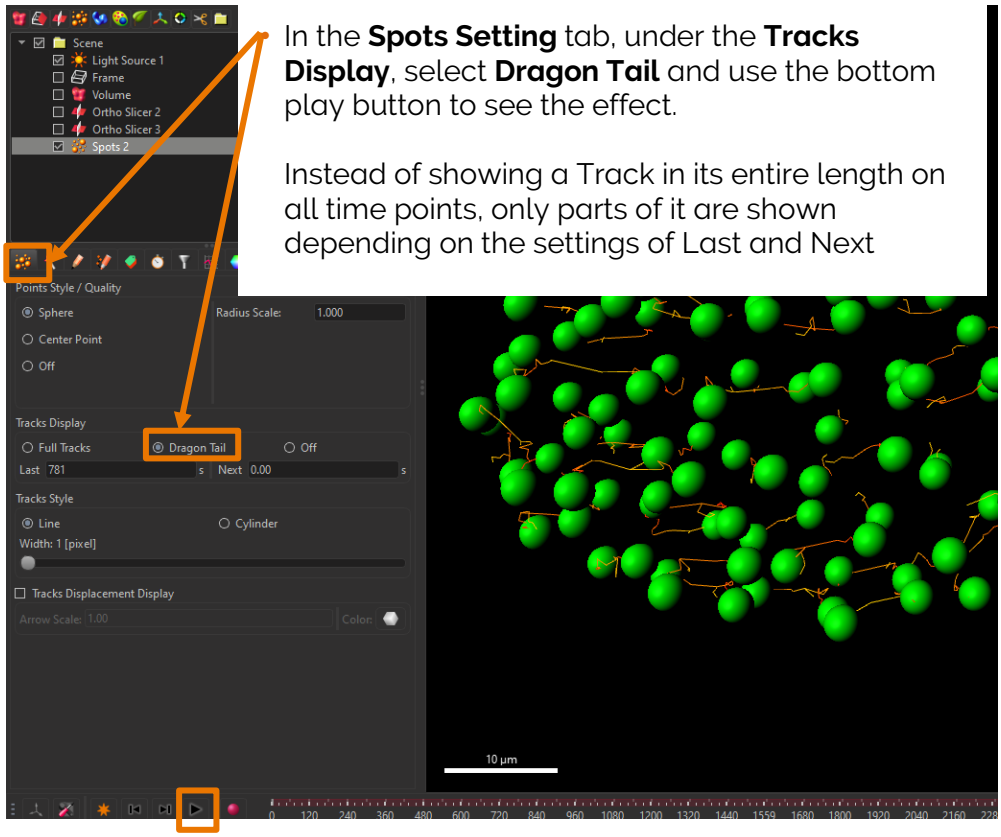

**Adjust the threshold of Track Duration to keep only correct tracks.**

! Think of using the bottom play button to stream the image forwards and backwards to verify the filtering impact

➢ Click on **the Green arrow** circled to finish the tracking

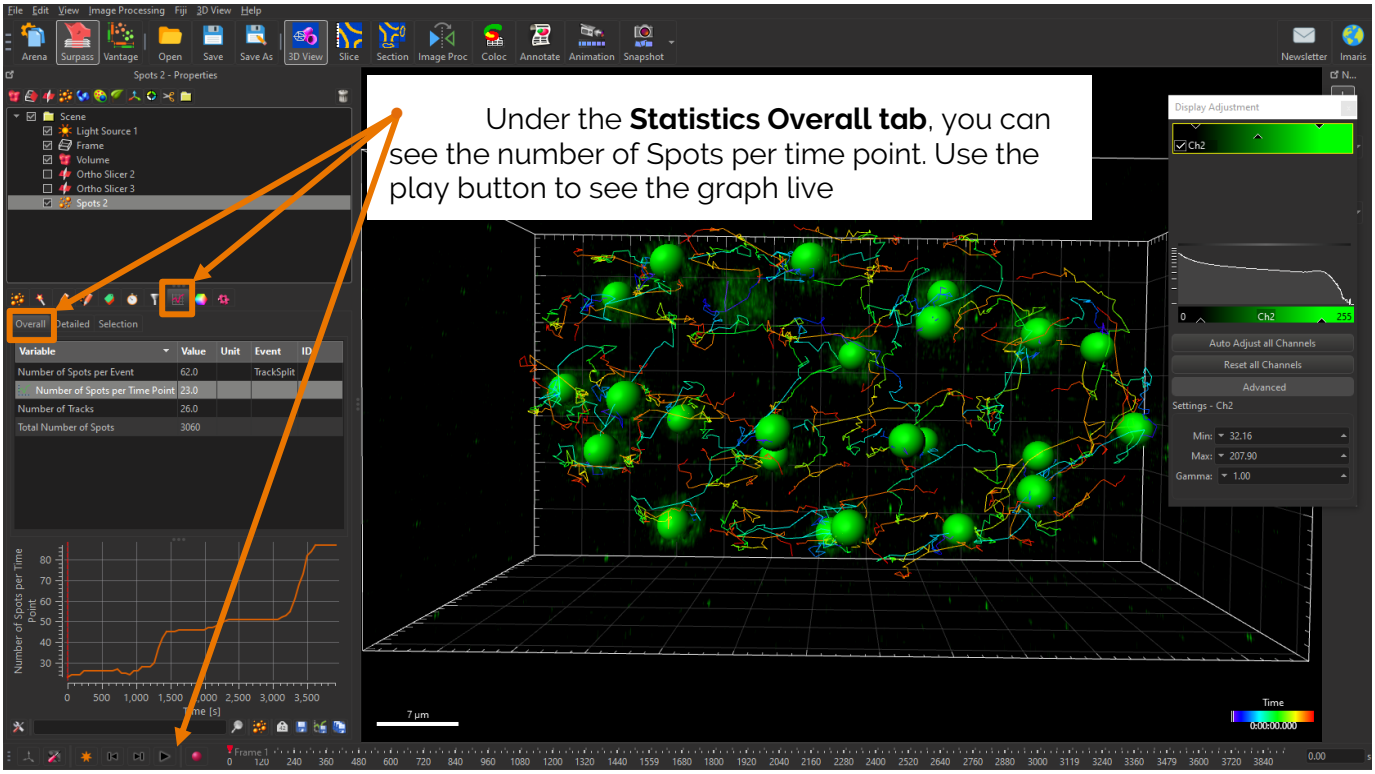
## Spots Tracking: Tracks Rendering

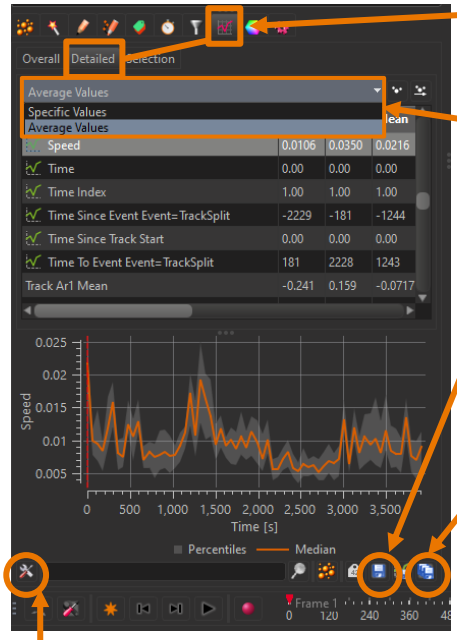
The Spots Tracking wizard of creation turns into a list of Property tabs 




In the **Spots Setting** tab, under the **Tracks Display**, select **Dragon Tail** and use the bottom play button to see the effect.

Instead of showing a Track in its entire length on all time points, only parts of it are shown depending on the settings of Last and Next

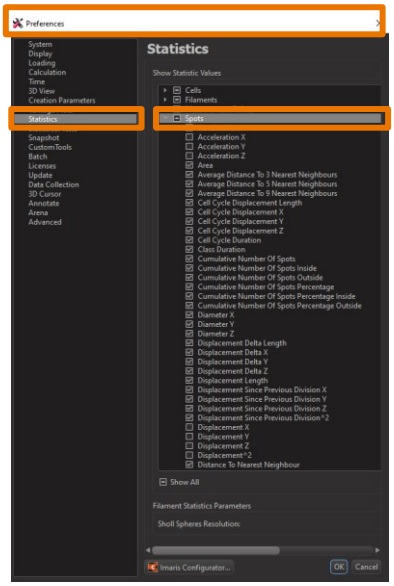



In the **Statistics Detailed tab,** using the play button, you can see the **Spots Average Speed** change over time (as Average Values was selected in opposition to Specific values)

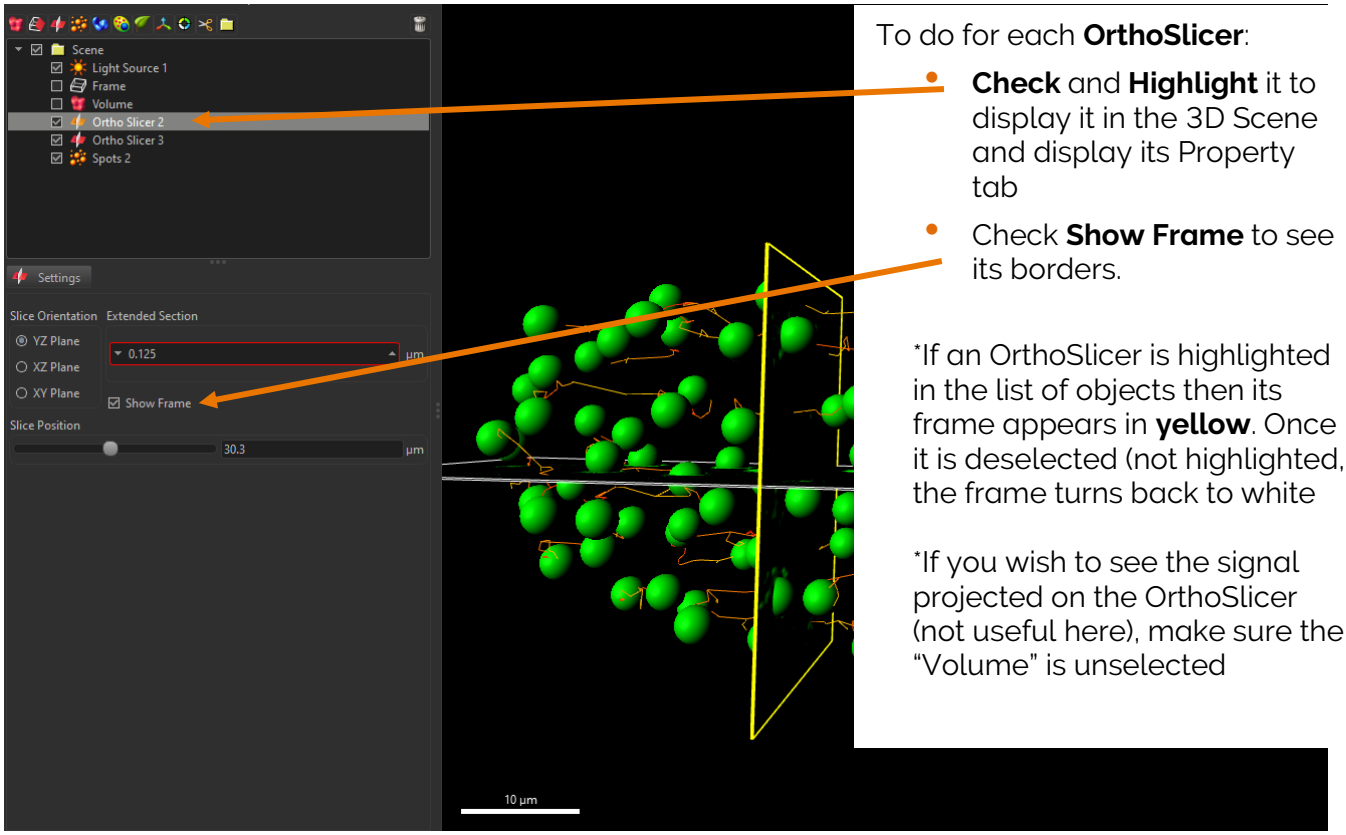
**Exports** only the **observed statistic** in the table

**Exports all** calculated **statistics** as preformatted .xls, .csv or xml file




Opens the **Preferences** window under the Spots Statistics where you can select or deselect any Statistic

Prepare the Spots Tracks visualisation for labelling



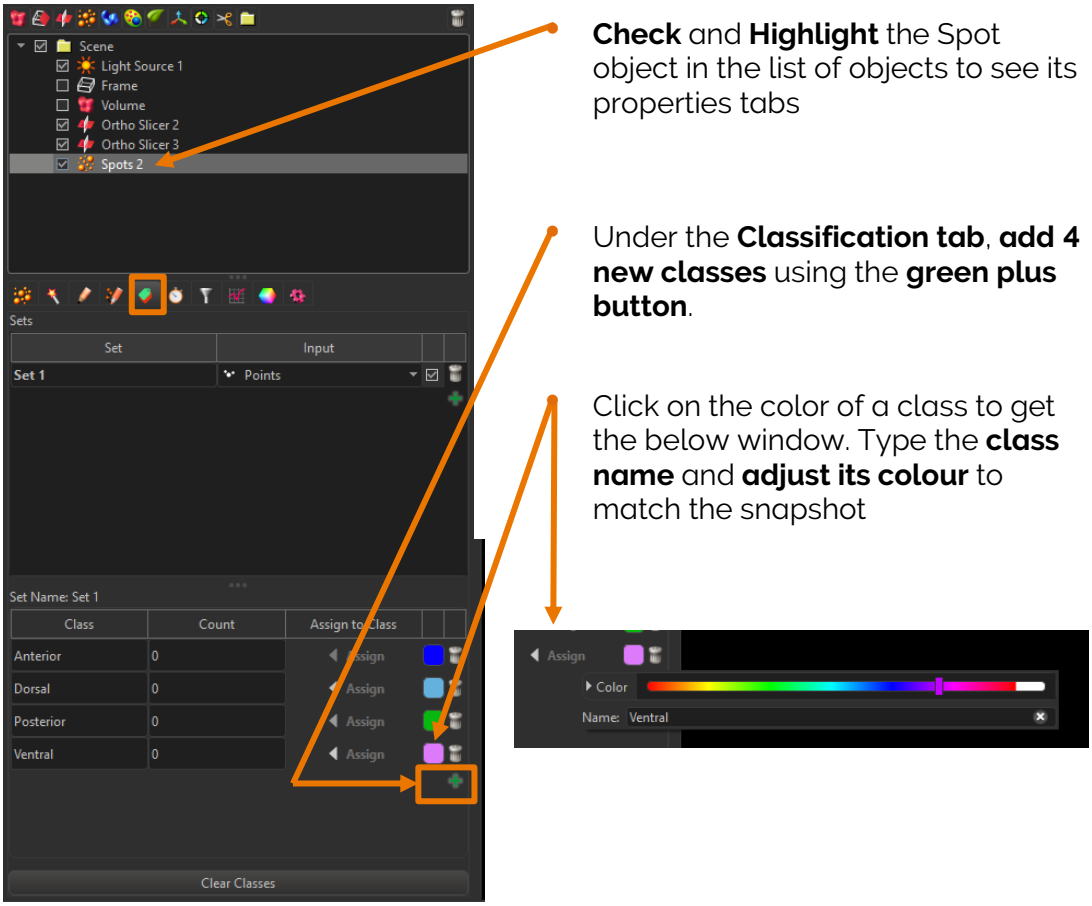

To do for each **OrthoSlicer**:

- **Check** and **Highlight** it to display it in the 3D Scene and display its Property tab
- Check **Show Frame** to see its borders.

*If an OrthoSlicer is highlighted in the list of objects then its frame appears in **yellow**. Once it is deselected (not highlighted, the frame turns back to white

*If you wish to see the signal projected on the OrthoSlicer (not useful here), make sure the "Volume" is unselected

Create the 4 Classes (labels) to be used

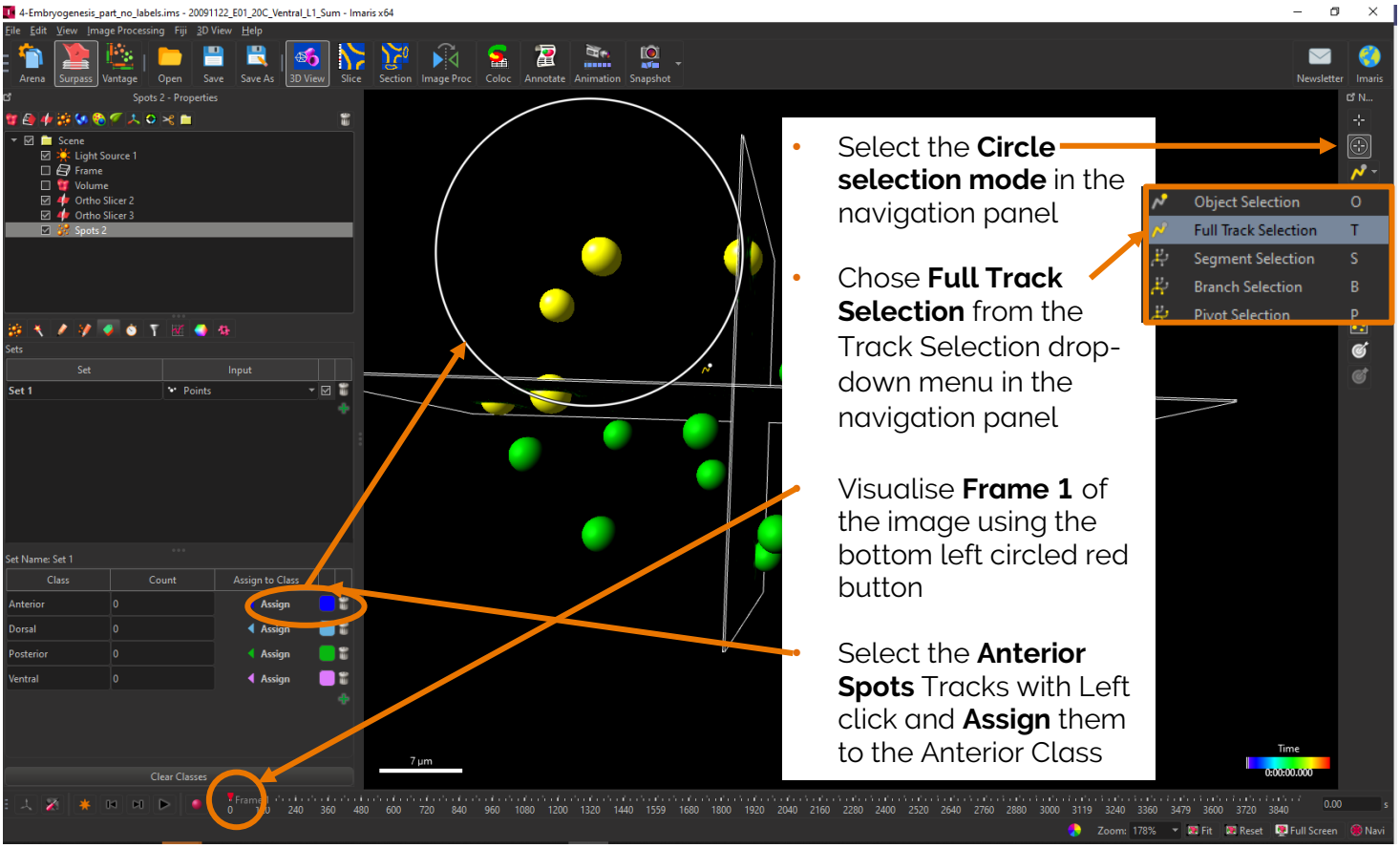



**Check** and **Highlight** the Spot object in the list of objects to see its properties tabs

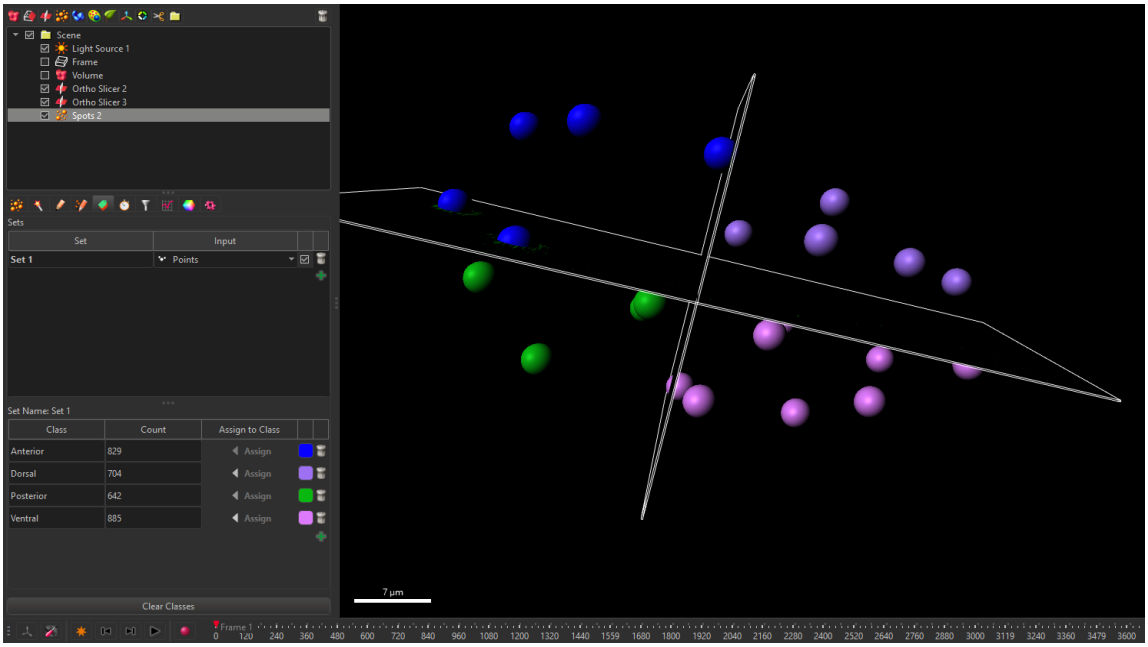
Under the **Classification tab**, **add 4 new classes** using the **green plus button**.

Click on the color of a class to get the below window. Type the **class name** and **adjust its colour** to match the snapshot

Manually label tracks as follow:

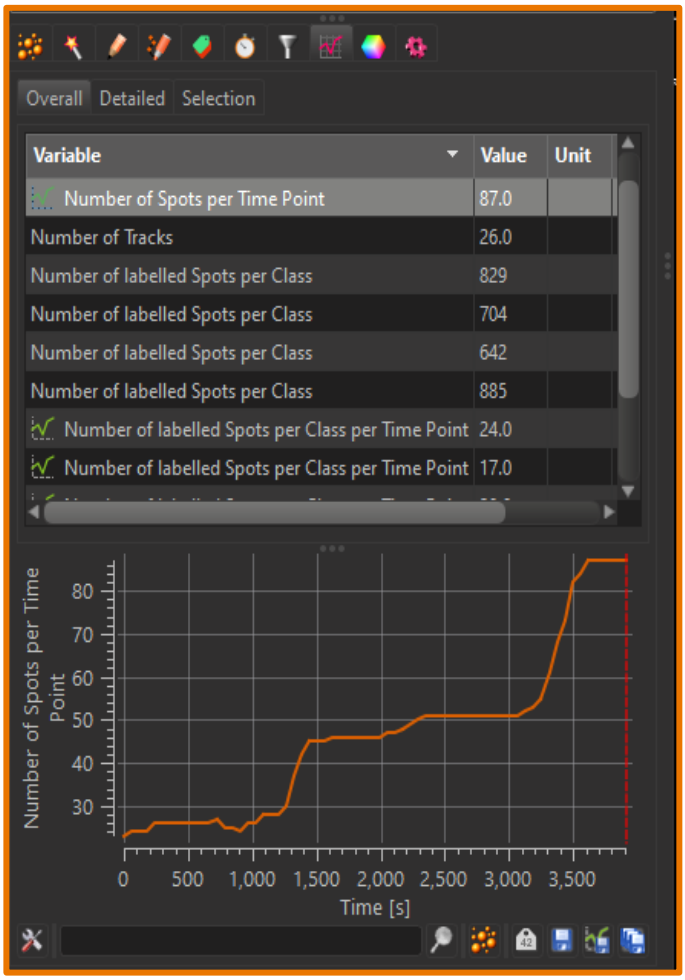


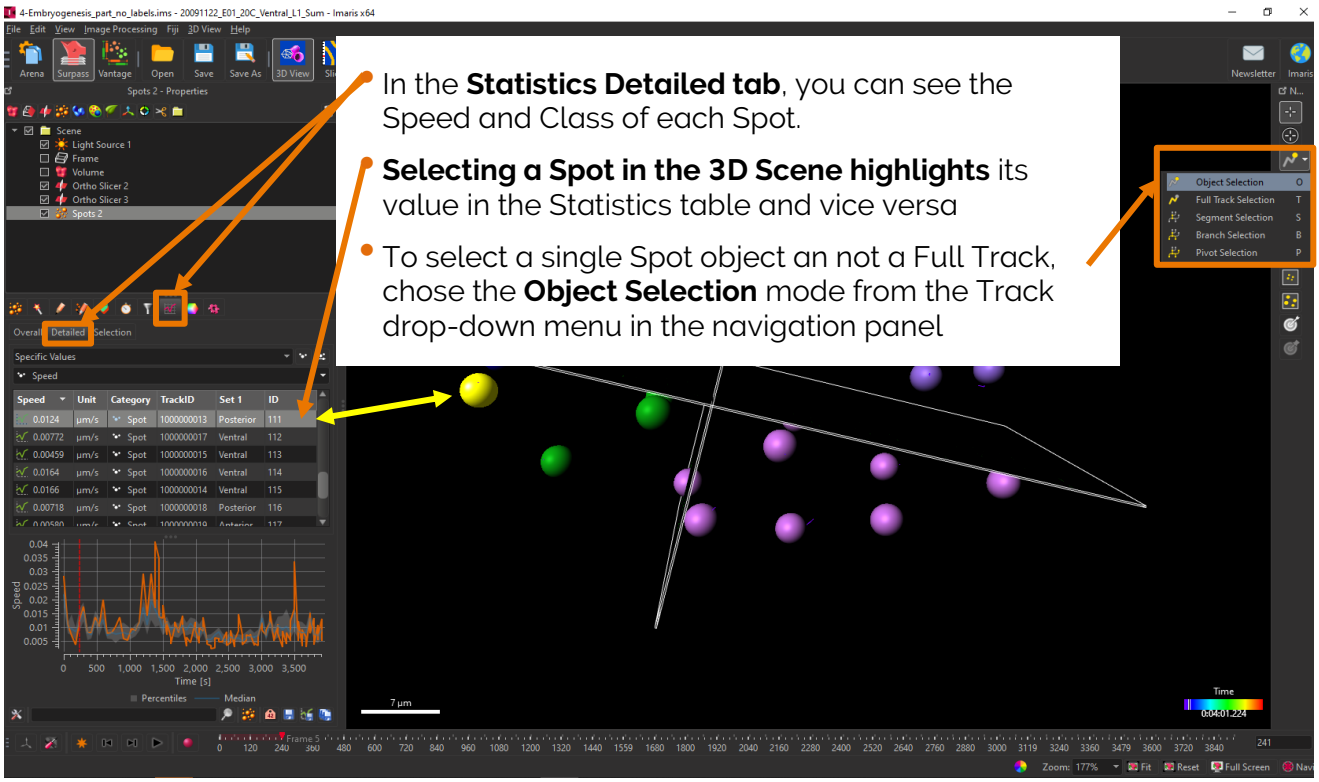

Repeat the process to Assign a Class to each Spot Track



NB: The class names are biological wrong. The location names are correct each for halve of the objects but not for a single quadrant. See https://en.wikipedia.org/wiki/Anatomical_terms_of_location#/media/File:Anatomical_Directions_and_Axes.JPG
I am not sure what would be correct tor the quadrants, maybe I should have used Quadrant1, Quadrant2,...

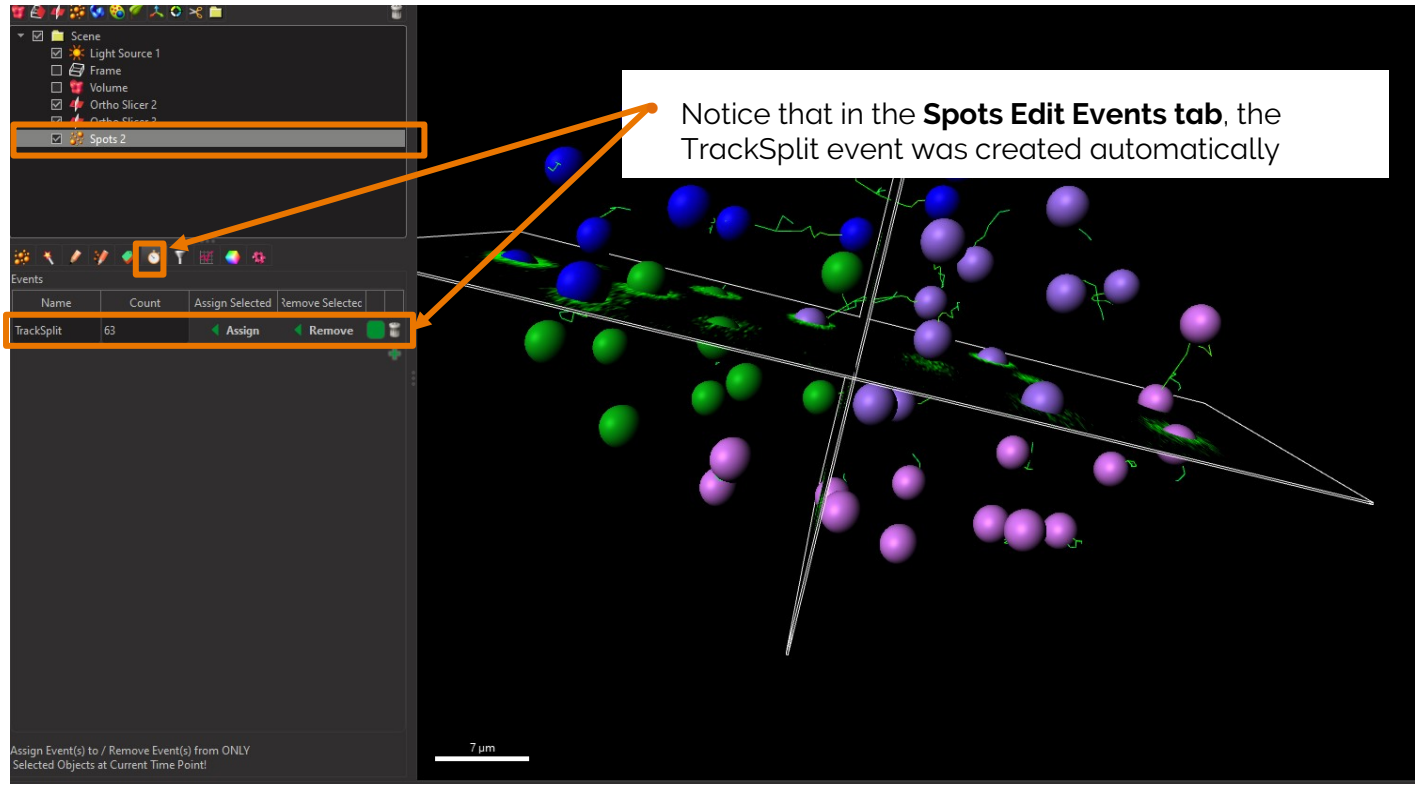
Now, in the Statistics **Overall tab**, you can see the number of Spots per Class



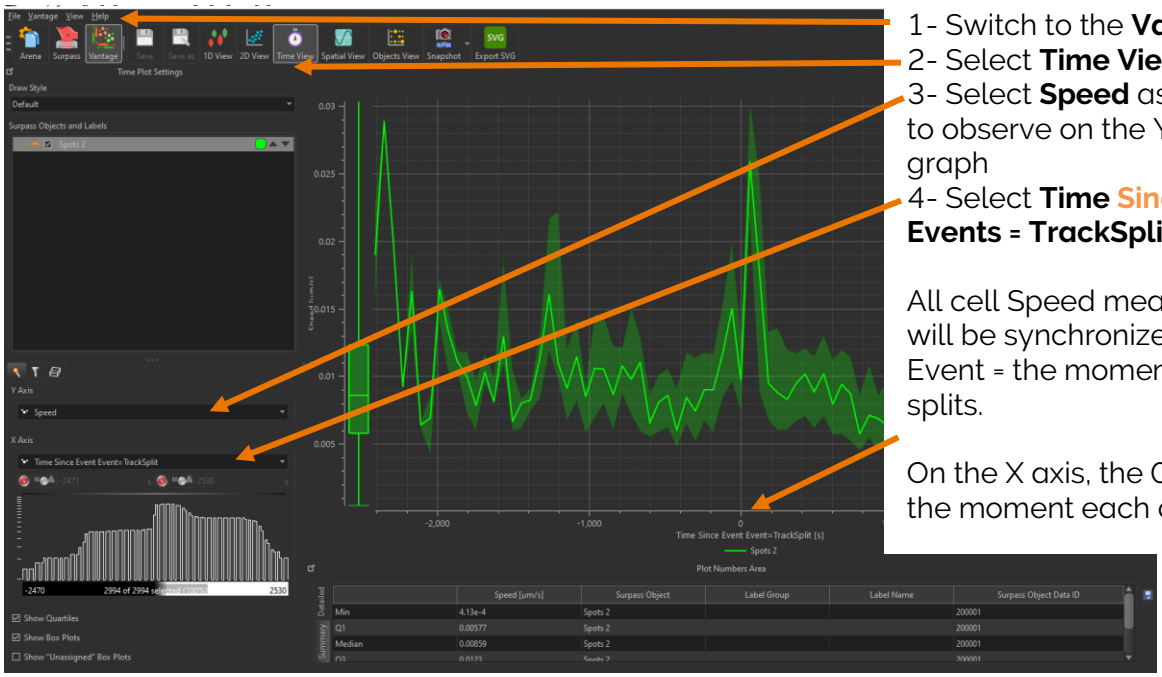



## Time Event parameter Set up: Spot Track Split

We will now proceed to the analysis of the behaviour of cells before and after they split. The event variable parameter for the following study will be set as being the moment a cell splits. This moment is different for each cell so it is a variable called here TrackSplit Event




## Time Event Plot: Speed & Intensity *vs.* Time Since Track Split Event

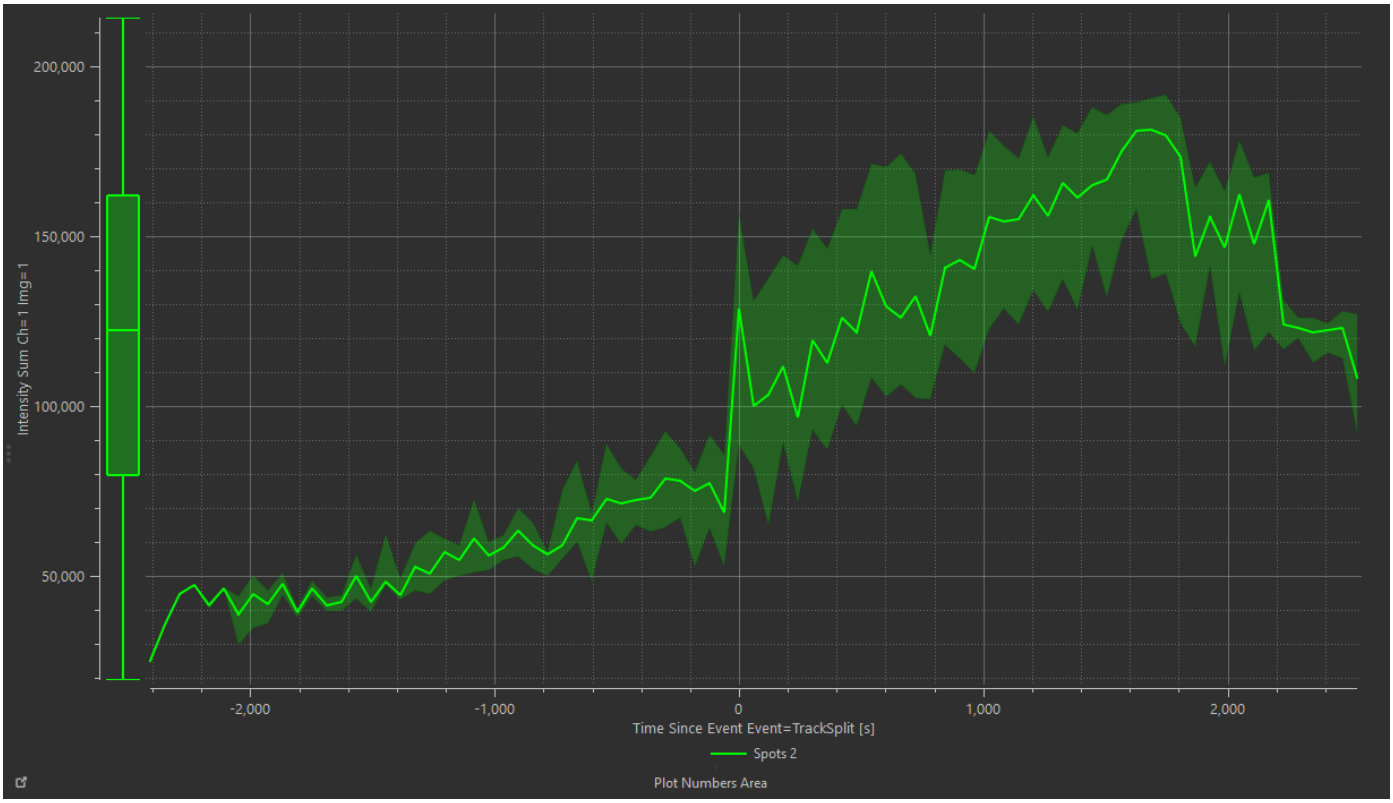



1- Switch to the **Vantage View**
2- Select **Time View**
3- Select **Speed** as a parameter to observe on the Y axis of the graph
4- Select **Time Since Event Events = TrackSplits**:

All cell Speed measurements will be synchronized on the Event = the moment each cell splits.

On the X axis, the 0 represents the moment each cell splits

In the Y axis, we see the speed of all cells before and after 0, so before and after each cell splits
The moment cell splits, there is an increase in their speed (see peak at X=0)




We can also observe for all cells, the differences in Intensity (Y) before and after cells split (X=0). It goes as expected, up and back down illustrating the DNA replication during the Cell Cycle

⇨ **Imaris facilitates the investigation of cell dynamics by providing an easy mechanism to synchronize measurements based on "events" independently defined for each cell. For example, in a movie of dividing cells where divisions happen at different times you can define the event as when a cell divides and then plot synchronized measurements in time relative to the event.**

More for later…
Imaris Tracking algorithms

# Killer T cell dynamic interacting with a cancer cell

## Exercise 5 - 10 mins

### Dataset description

**File: 5-Immune_Cell_Attacking_Cancer_Cell.ims**

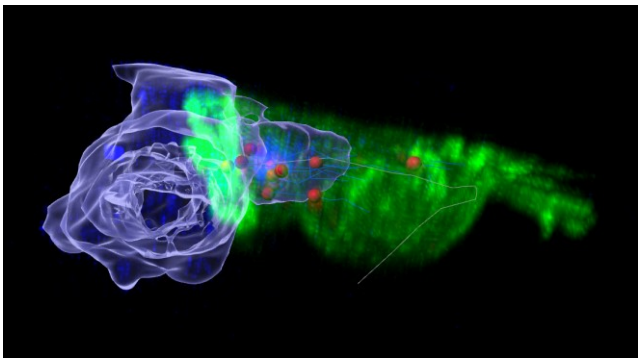
Channel 1 : Cancer Cell
Channel 2 : Killer T-Cell
Channel 3 : Cytotoxic Granules

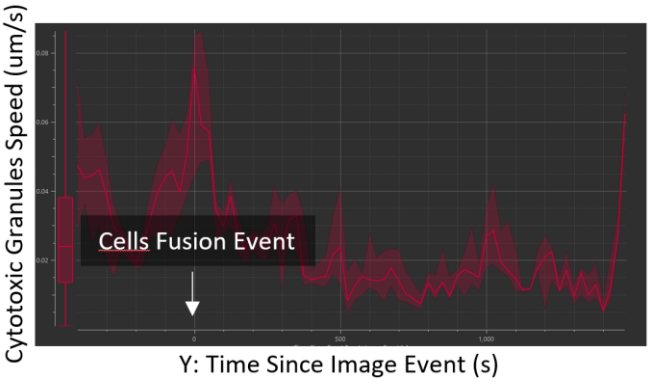
3D time lapse image of a Killer T cell eating a cancer cell,
Courtesy of Dr. Alex Ritter, Cambridge University, UK

### Scientific question and complete image analysis protocol

**How do cytotoxic granules behave when immune killer cells contact a cancer cell?**

1- Create and track Surfaces for the Cancer Cell and the Killer T Cell nuclei (Ch 1 Blue)
2- Create and track Spots for the Cytotoxic Granules (Ch 3 Red)
3- Set manually as Image Time Event, the time point when the Killer T Cell and Cancer Cell contact
4- Plot the Cytotoxic Granules (Spots) Speed vs. Time relative to the contact event to detect any Speed changes

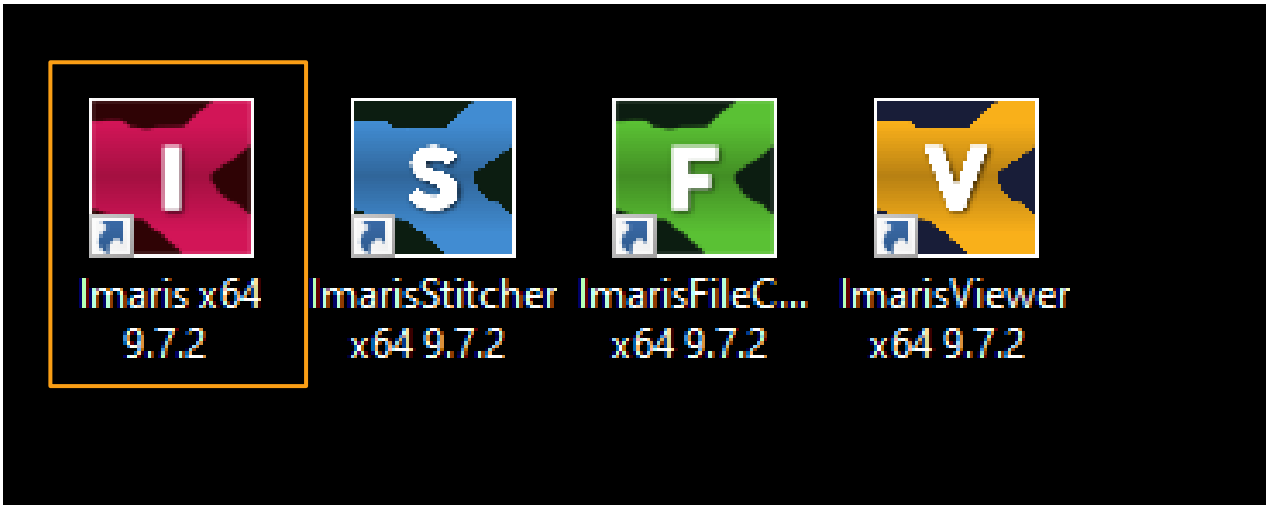





### Step by step guided exercise

#### Open the image file:

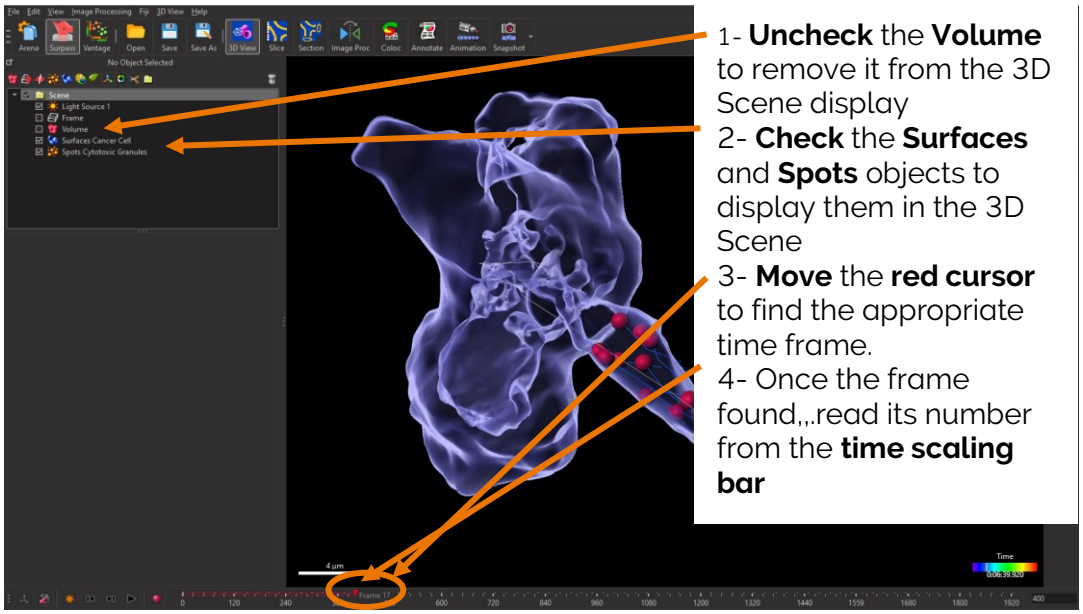
Open Imaris by double clicking on the main Imaris pink application icon:



Make sure you are in the "Arena" interface which is Imaris library of images and double click on the file **5-Immune_Cell_Attacking_Cancer_Cell.ims**

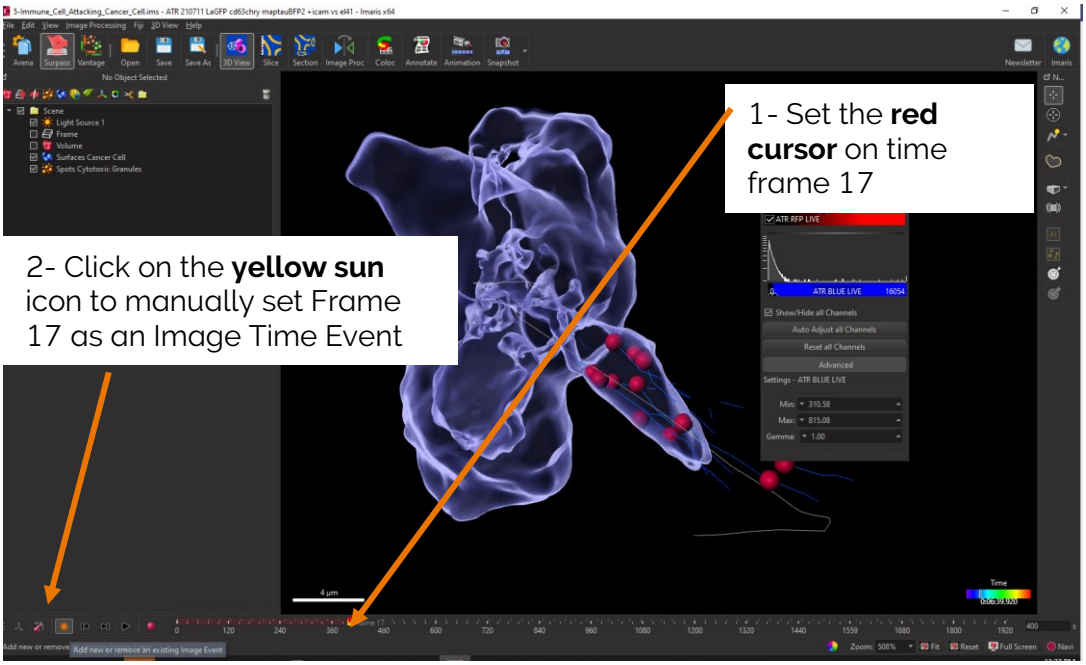

## Time Event Set up: Image Time Frame

Watch the image time lapse using the bottom left play button. Try to identify the moment (frame number) when the Killer T Cell and the Cancer Cell gets in contact so that the Cytotoxic Granules can cross from one cell to the other.



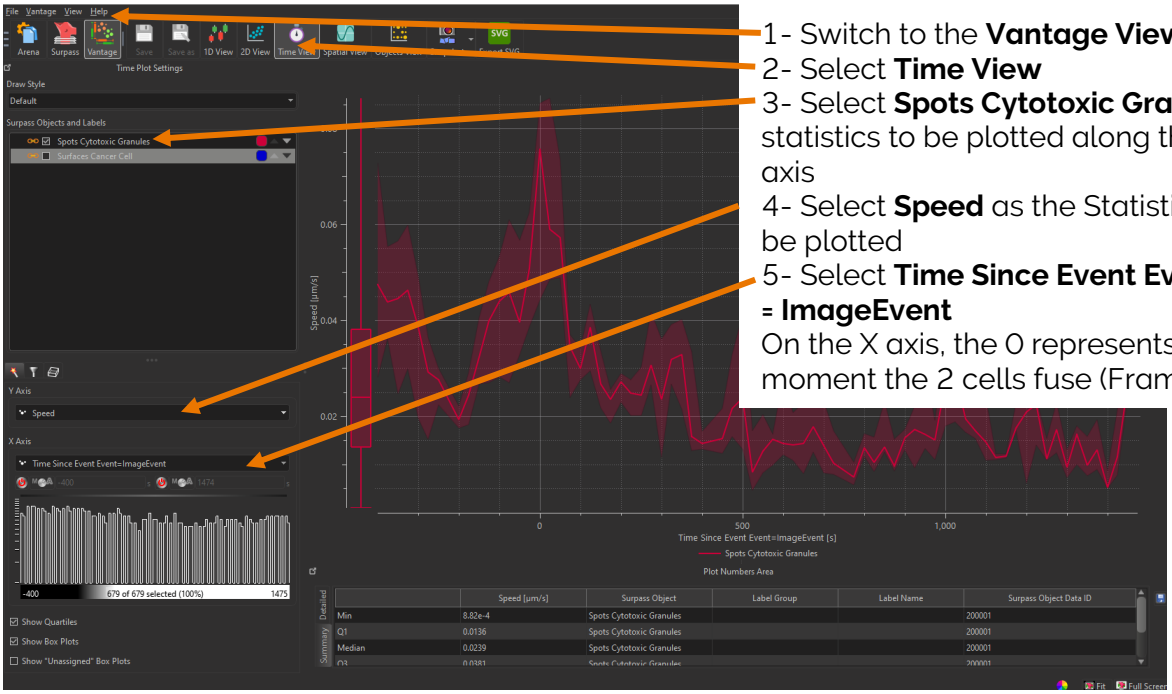

1- **Uncheck** the **Volume** to remove it from the 3D Scene display
2- **Check** the **Surfaces** and **Spots** objects to display them in the 3D Scene
3- **Move** the **red cursor** to find the appropriate time frame.
4- Once the frame found,,.read its number from the **time scaling bar**

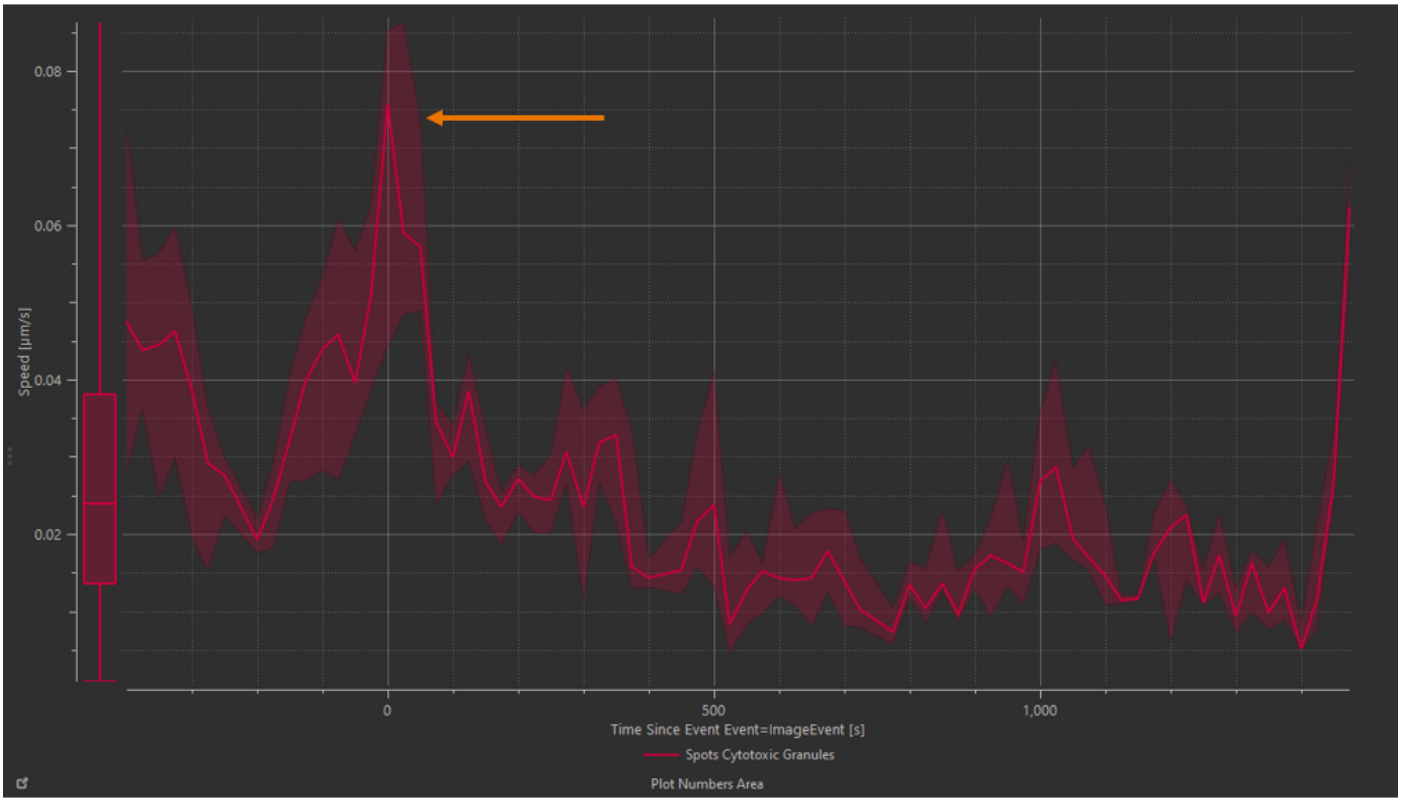
Set manually an Image Time Event at time point 17




## Time Event Plot: Speed *vs.* Time Since Image Time Frame

Plot the Cytotoxic Granule Spot Speeds vs. Time relative to the contact event to detect any Speed changes before and after the fusion of the Killer T cell and the Cancer cell






There is a speed increase (peak) of the Cytotoxic Granules when they enter the Cancer Cell (X=0)

## More for later...about this topic:.

Time plots

# Batch 3D genomic analysis by DNA FISH
## Exercise 6 - 45 mins

### Dataset description

**Folder 6-Batch Colocalized Spots** which includes:

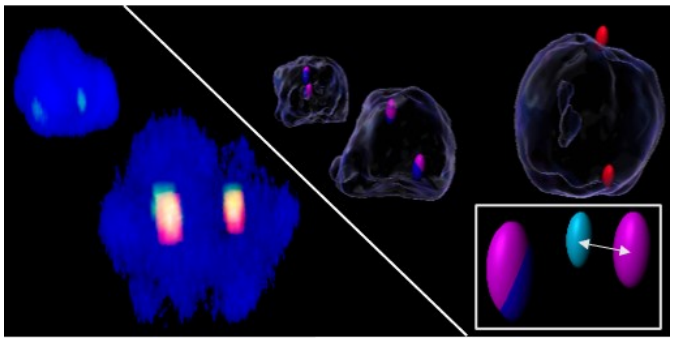
- Folder **Experiment1** : duplicates images **Experiment1.1.ims** & **Experiment1.2.ims**
- Folder **Experiment2** : duplicates images **Experiment2.1.ims** & **Experiment2.2.ims**
- **Coloc.icsx**: Batch protocol parameters
- **Experiment1and2Comparison.iplx**: comparison plot of the 4 images

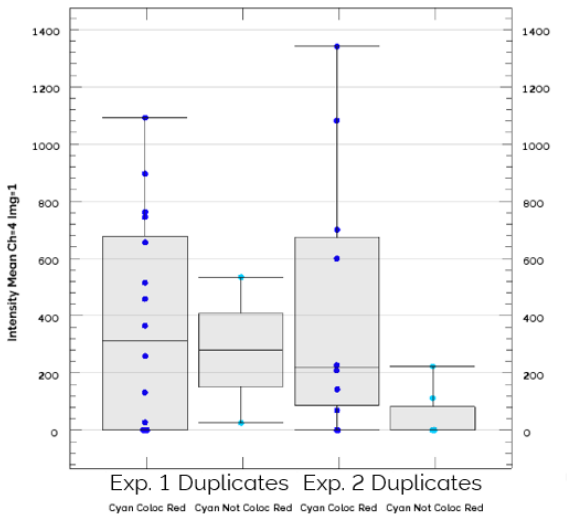
Murine stem cells with 3 probes and DAPI.
Courtesy of Dr. Julie Chaumeil and Dr. Pierre Bourdoncle, Institut Cochin, Paris, France

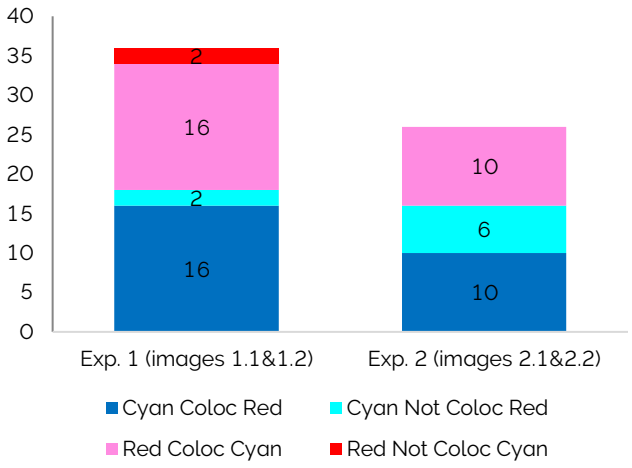
### Scientific question and complete image analysis protocol

**Do two types of alleles (Red and Cyan) colocalize and if yes to which extent? Analysis to be repeated and normalized on multiple images so to be run in batch.**

1- Set-up a single Batch Protocol that:
   a. Detects 2 types of alleles (Cyan & Red) as elliptical Spots
   b. Detects all nuclei as Surfaces
   c. Calculates inter-alleles distances (Cyan - Red distances)
   d. Classify Cyan and Red alleles as Colocalizing or Not colocalizing using an inter-allele distance threshold
2- Launch the batch protocol on folders and sub-folders of images
3- Visualize results in a plot
4- Export statistics in excel for further statistical analysis



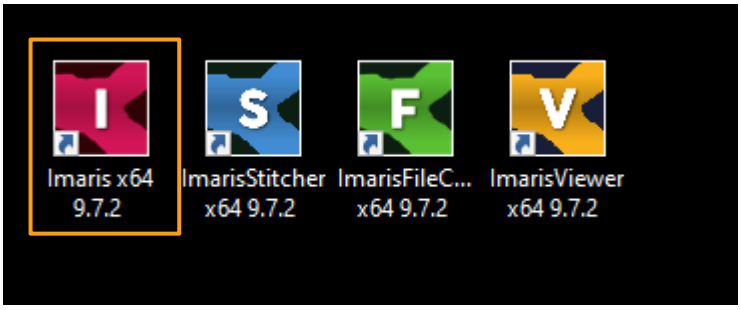


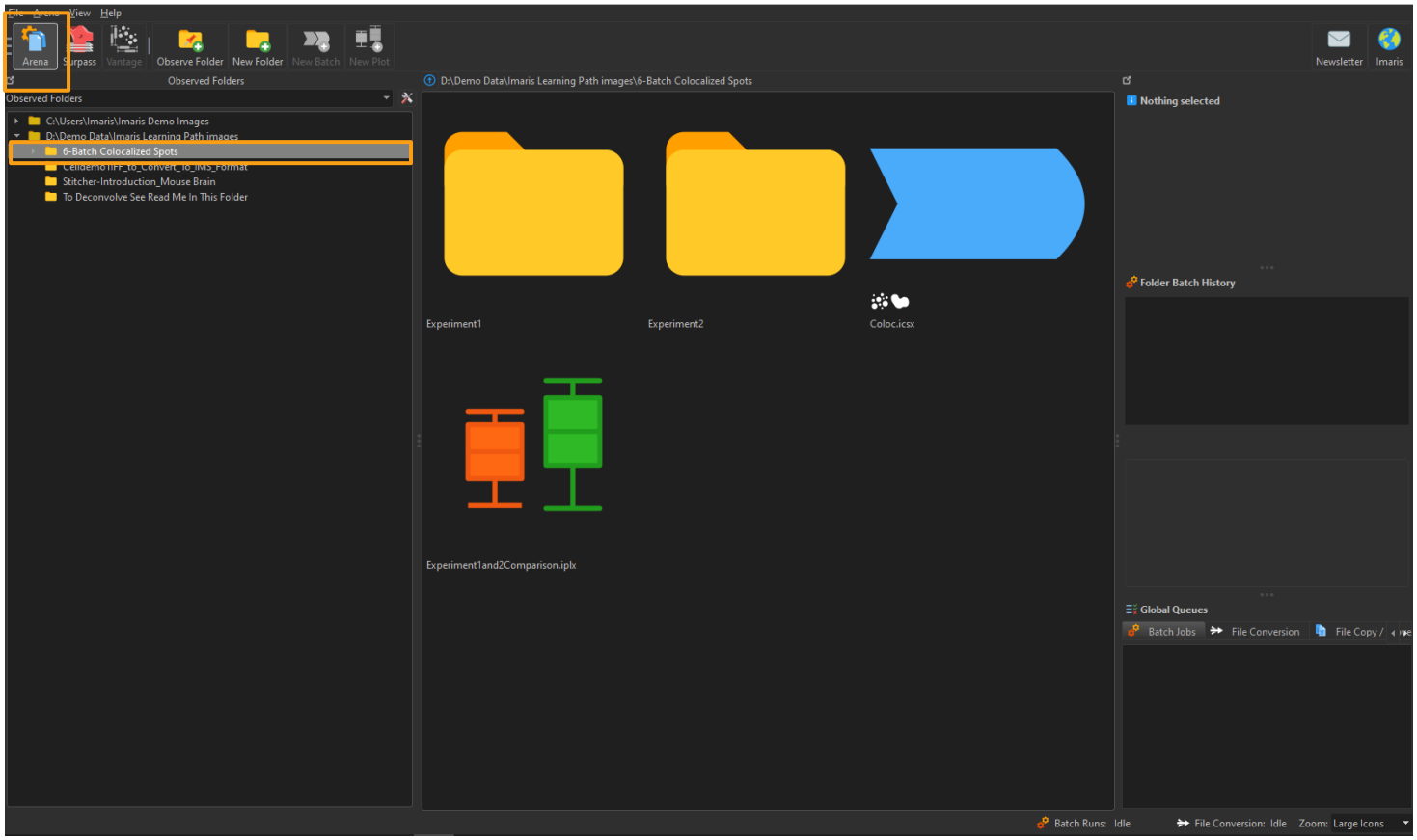
## Step by step guided exercise

This exercise was already solved. A batch protocol was set-up & ran and a plot was already created for results visualisation.
First, we will explore all the created files in the Arena interface before going to the actual exercise where we'll see how to create a batch protocol, launch it and visualize the results.

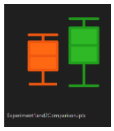
Open Imaris by double clicking on the main Imaris pink application icon:




Make sure you are in the "Arena" interface which is Imaris library of images and under the Batch exercise image folder : 6-Batch Colocalized Spots

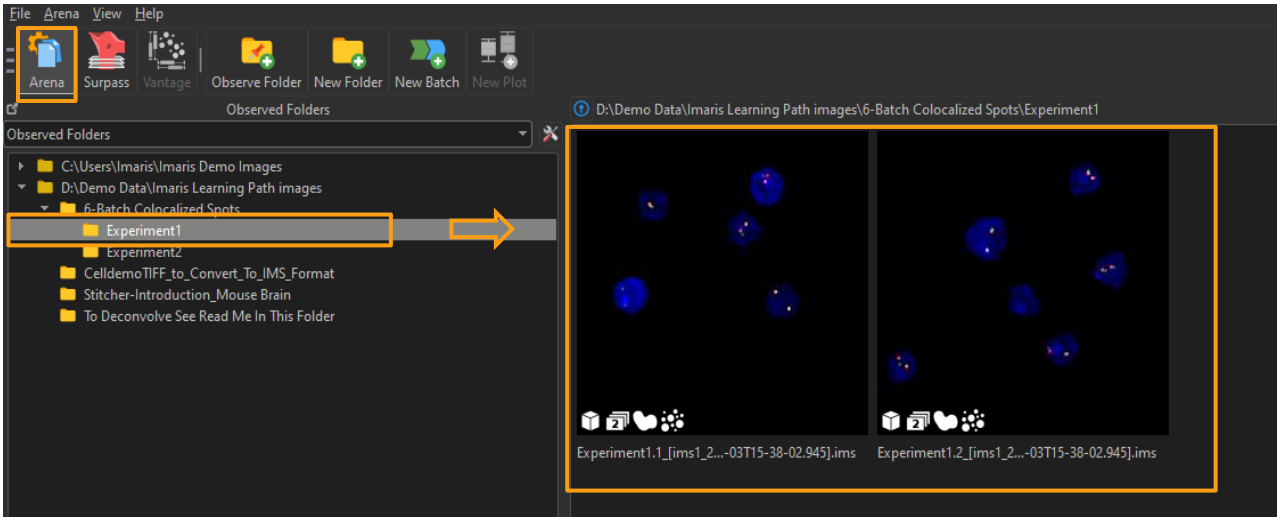


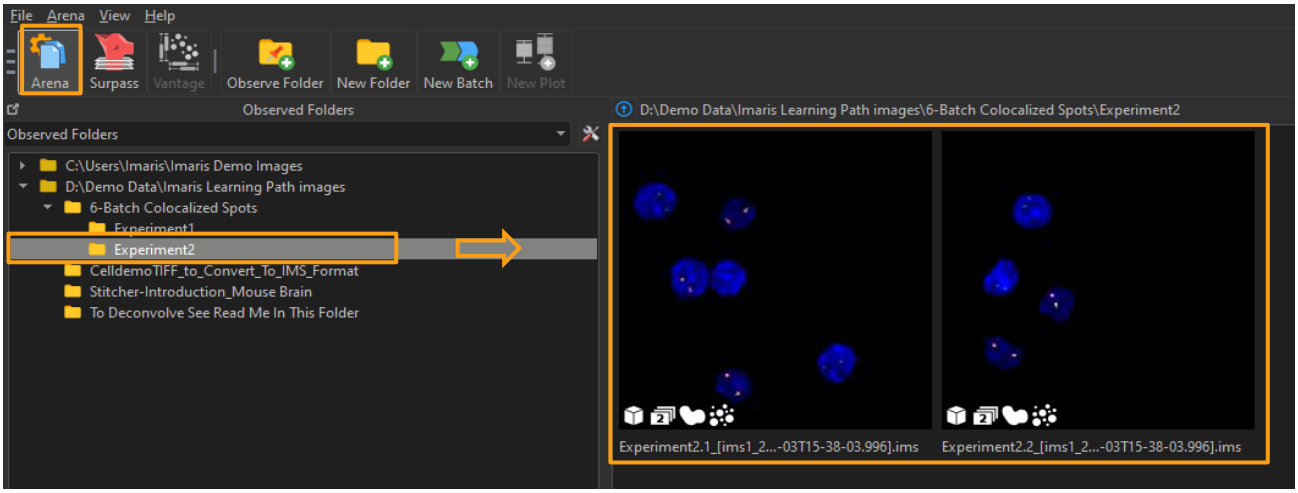

This blue icon leads to saved parameters for a batch protocol analysis



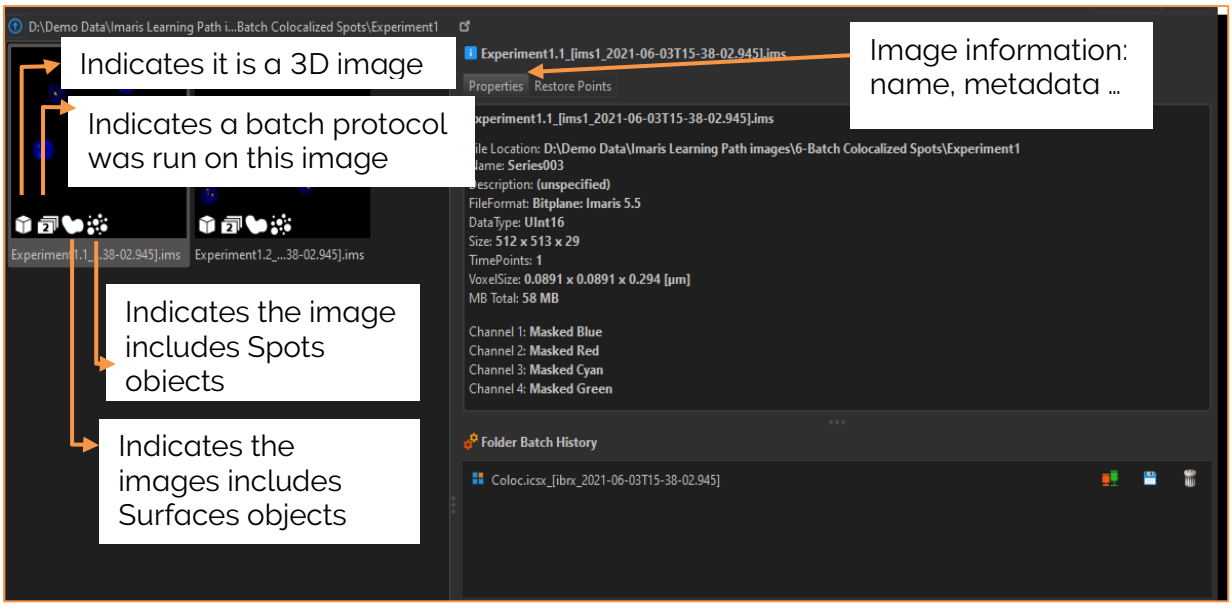
The bicoloured boxplot icon leads to the Vantage view for statistical plotting such as a comparison plot

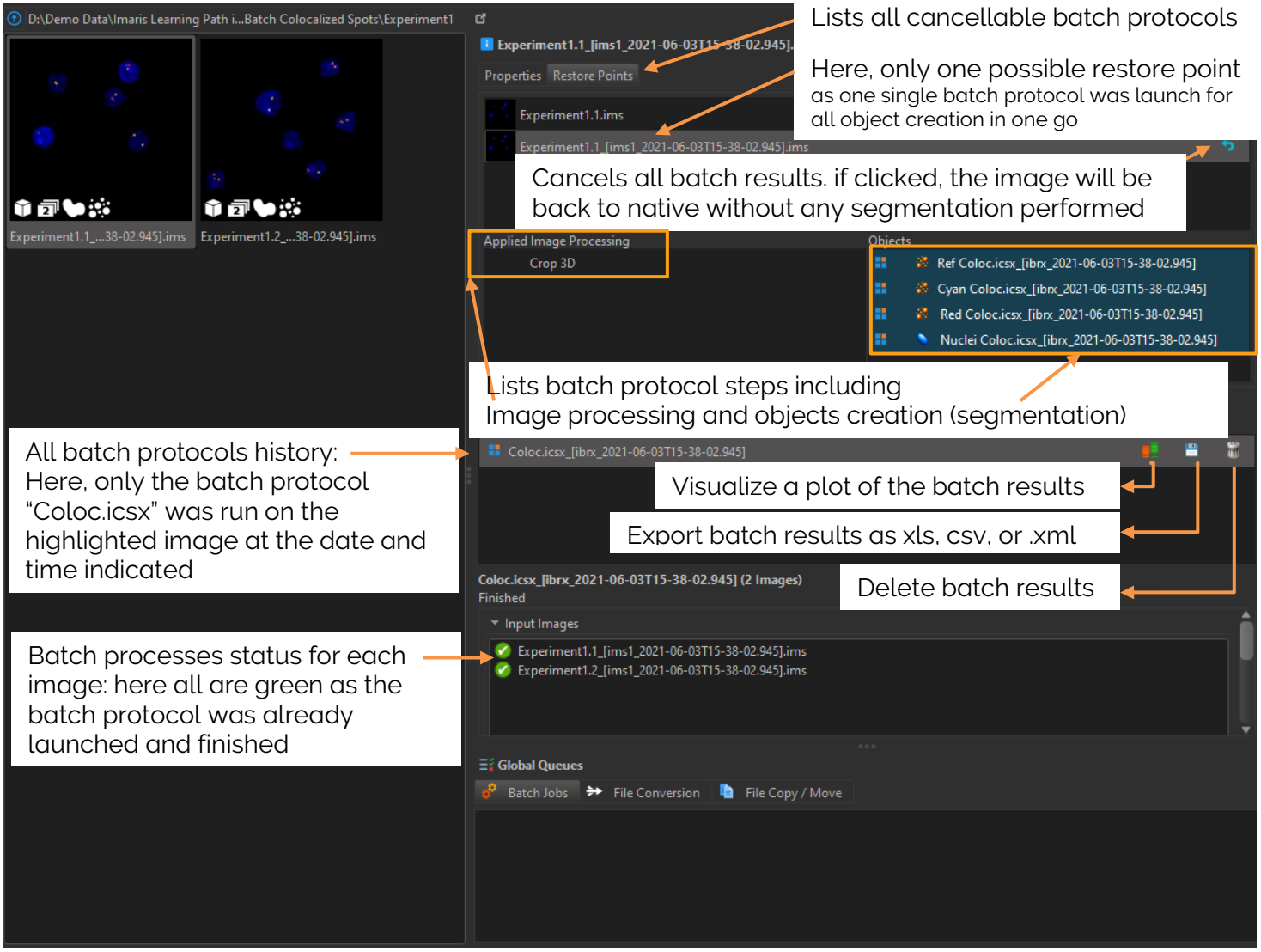
Highlight successively the Experiment1 and Experiment2 folders to explore them. Each folder contains duplicate images of 2 different experiments:




Highlight (single click) one image to see the following information:



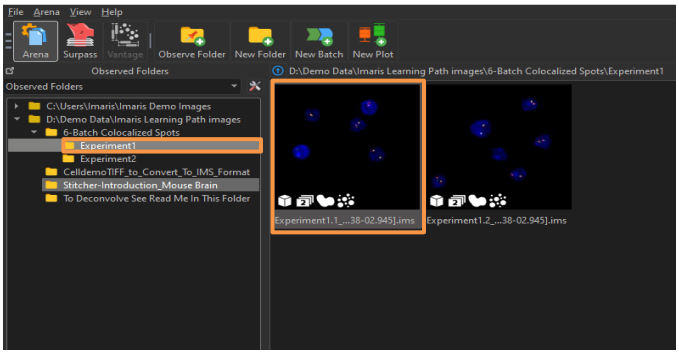



## Typical image exploration

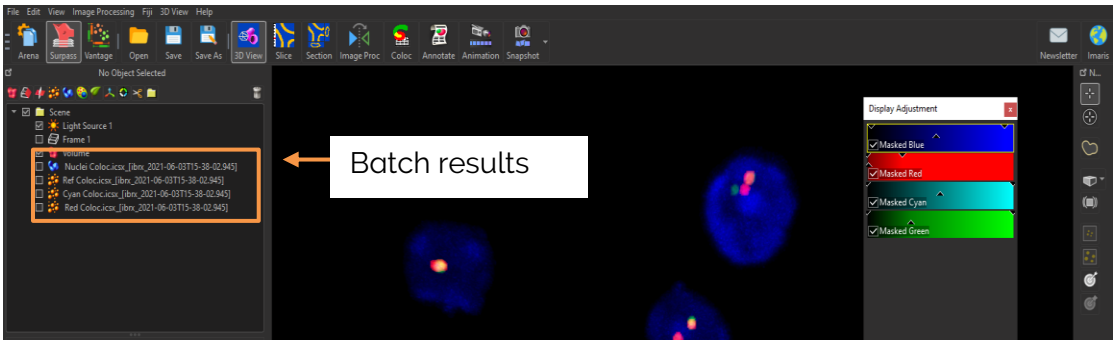
Before setting-up a Batch protocol, one should have already explored key image properties from different conditions such as a negative control, a positive control and an unknown case.

After this exploration, one has a good idea of how to set-up segmentation parameters to batch like: which detection tool to use (Spots, Surfaces, Cell or FilamentTracer), which algorithms to use, structure shapes and sizes, resolution etc ….

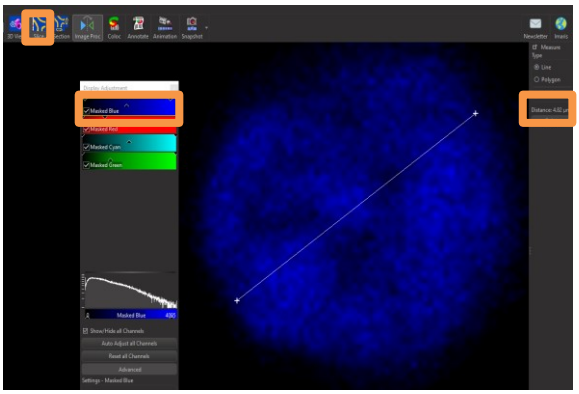
Therefore, in this first step, you can explore one of the four images to batch. Double click on the Eperiment1/Experiment1.1.ims file to open it in the Surpass 3D interface:

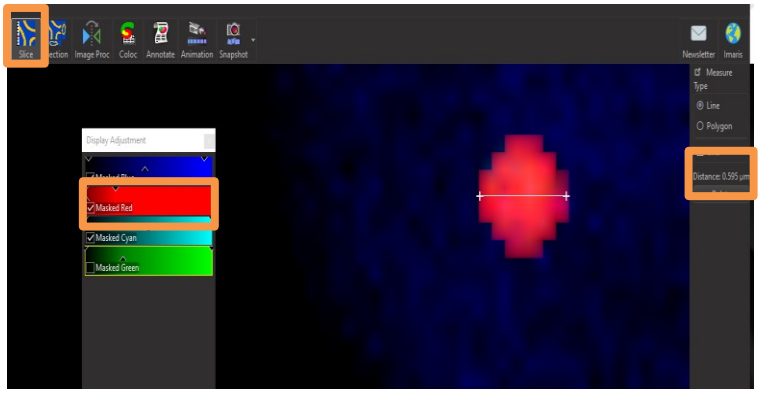


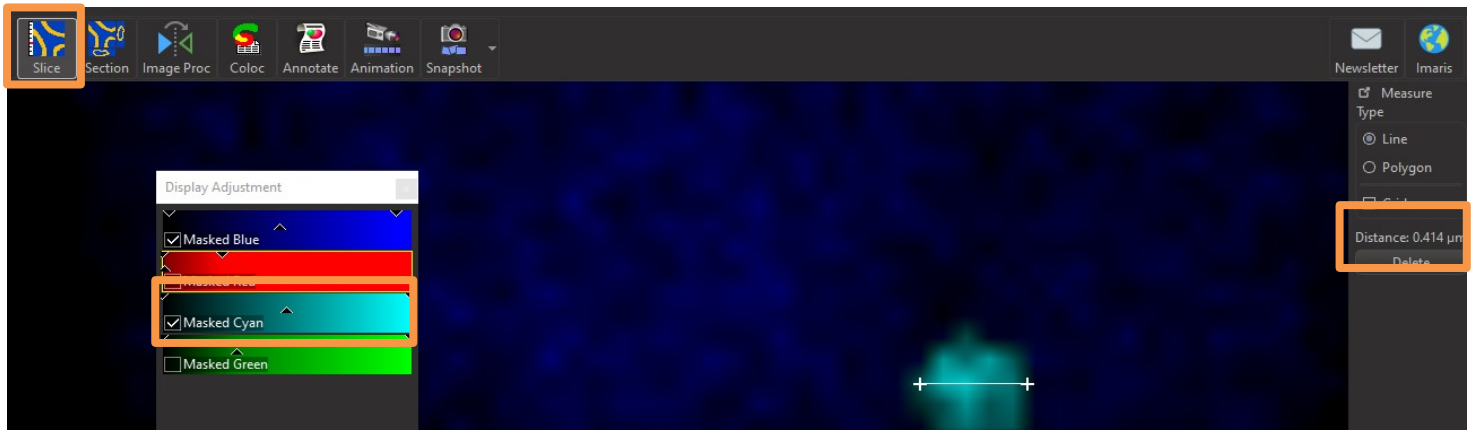
As all files were already batch processed, batch protocol results will appear, meaning you will see already created objects.



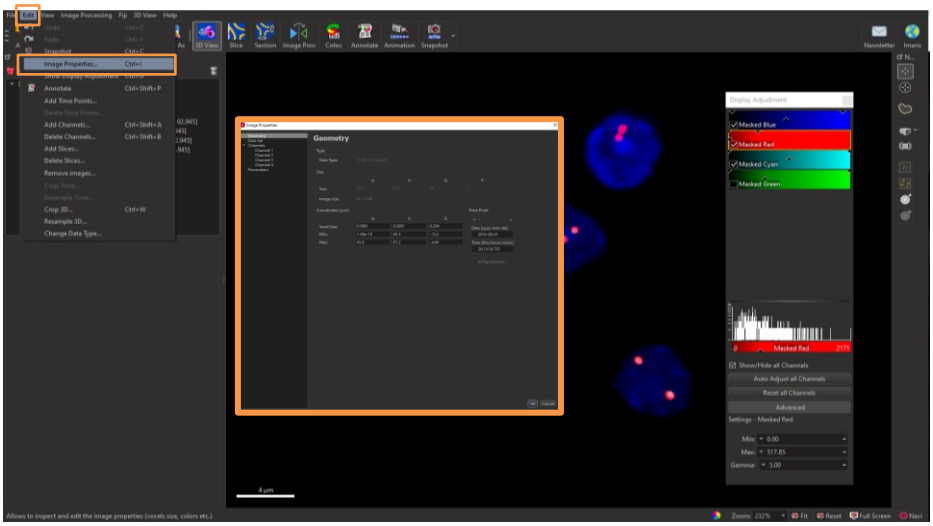

- If you wish, to learn how to measure quickly distances to explore structure sizes, click on this button 



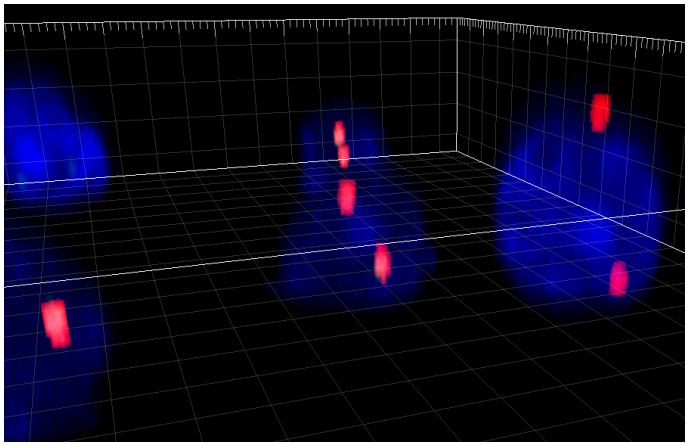




- The image resolution window opens with the Ctrl + I command if you wish to check it



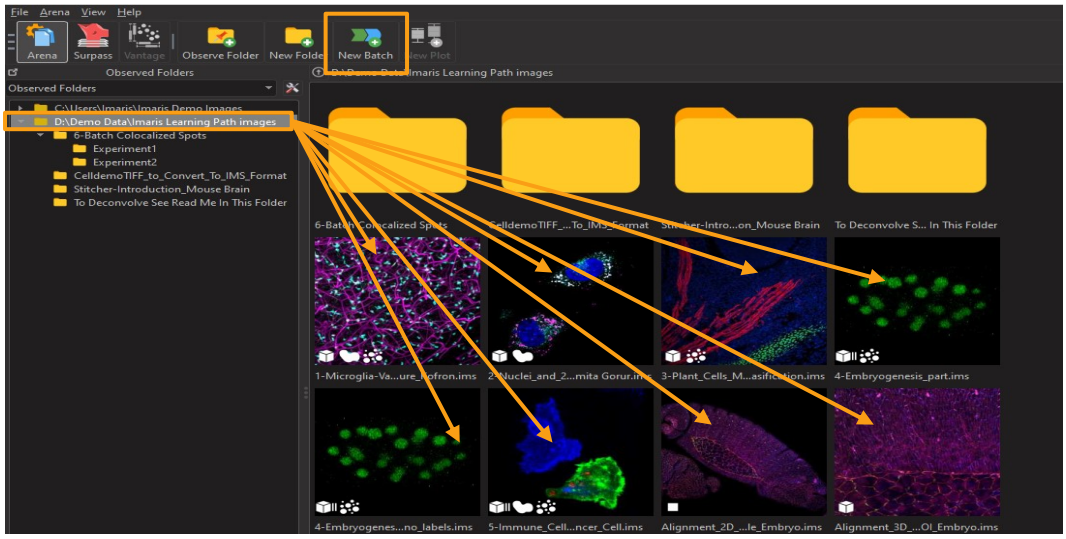
Note that the image is stretched along the Z direction. Alleles don't look spherical but rather elliptical:



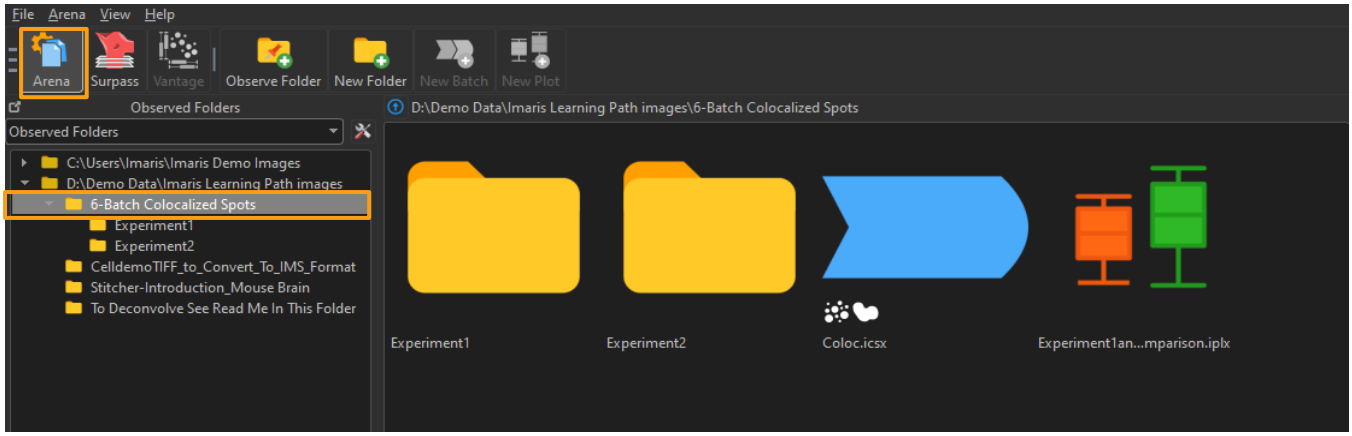
This is due to the diffraction of the light inherent to any microscope . This deformation could be corrected by a deconvolution image processing method but that could be another exercise …

## Batch Editor

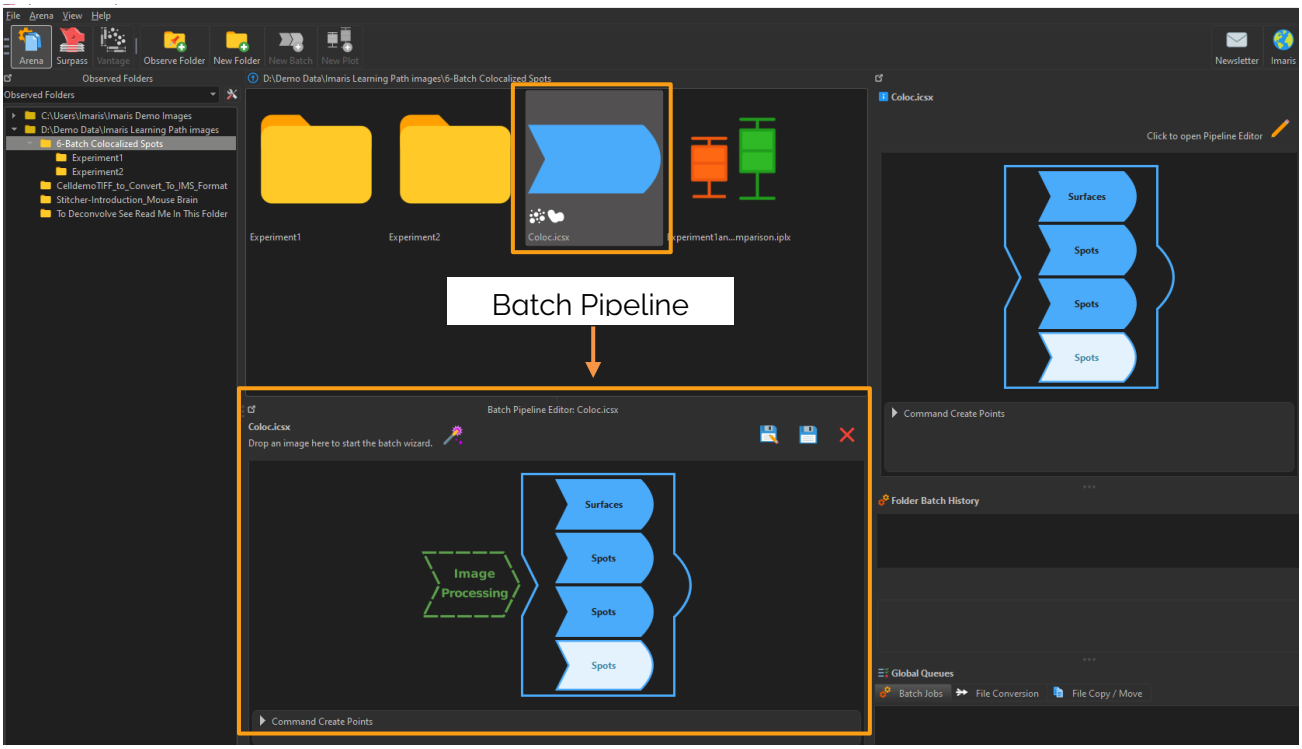
To create a new batch protocol, you need to be in a folder that contains images so the "New Batch" blue and green icon becomes clickable. Do not create a new Batch protocol.



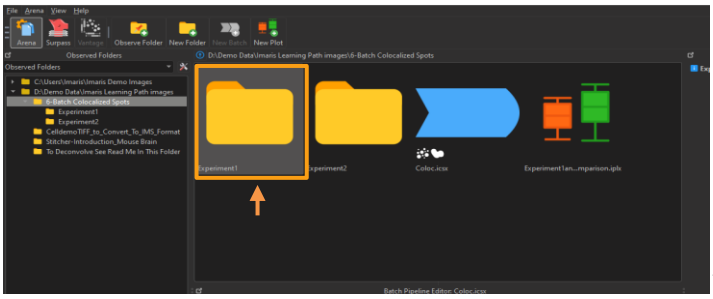
As a batch protocol was already created for this exercise, instead of creating a new batch protocol from scratch, we will explore all steps of the existing one. To do so, first move to the batch exercise folder as follow:

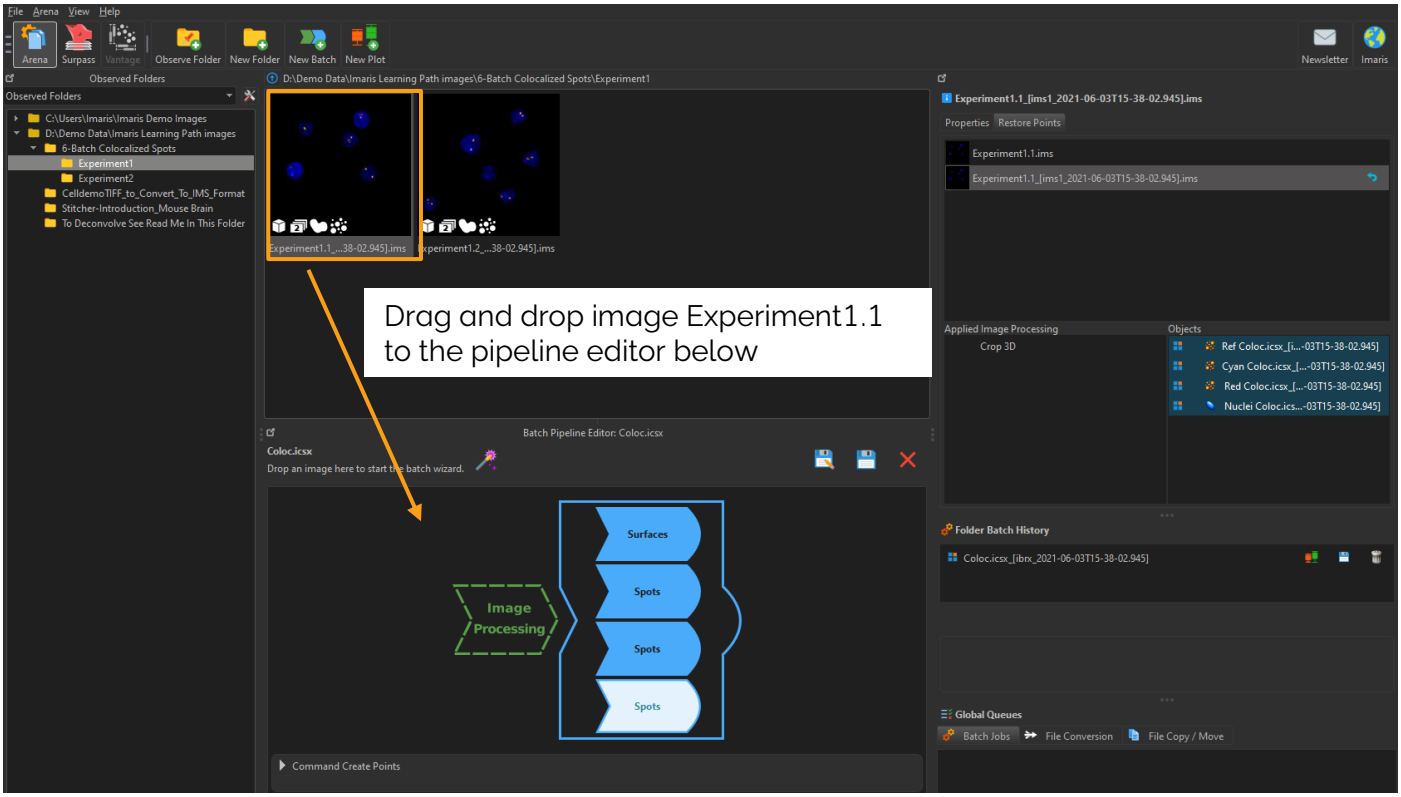



Then double click on the batch parameter blue icon to open the batch pipeline editor:

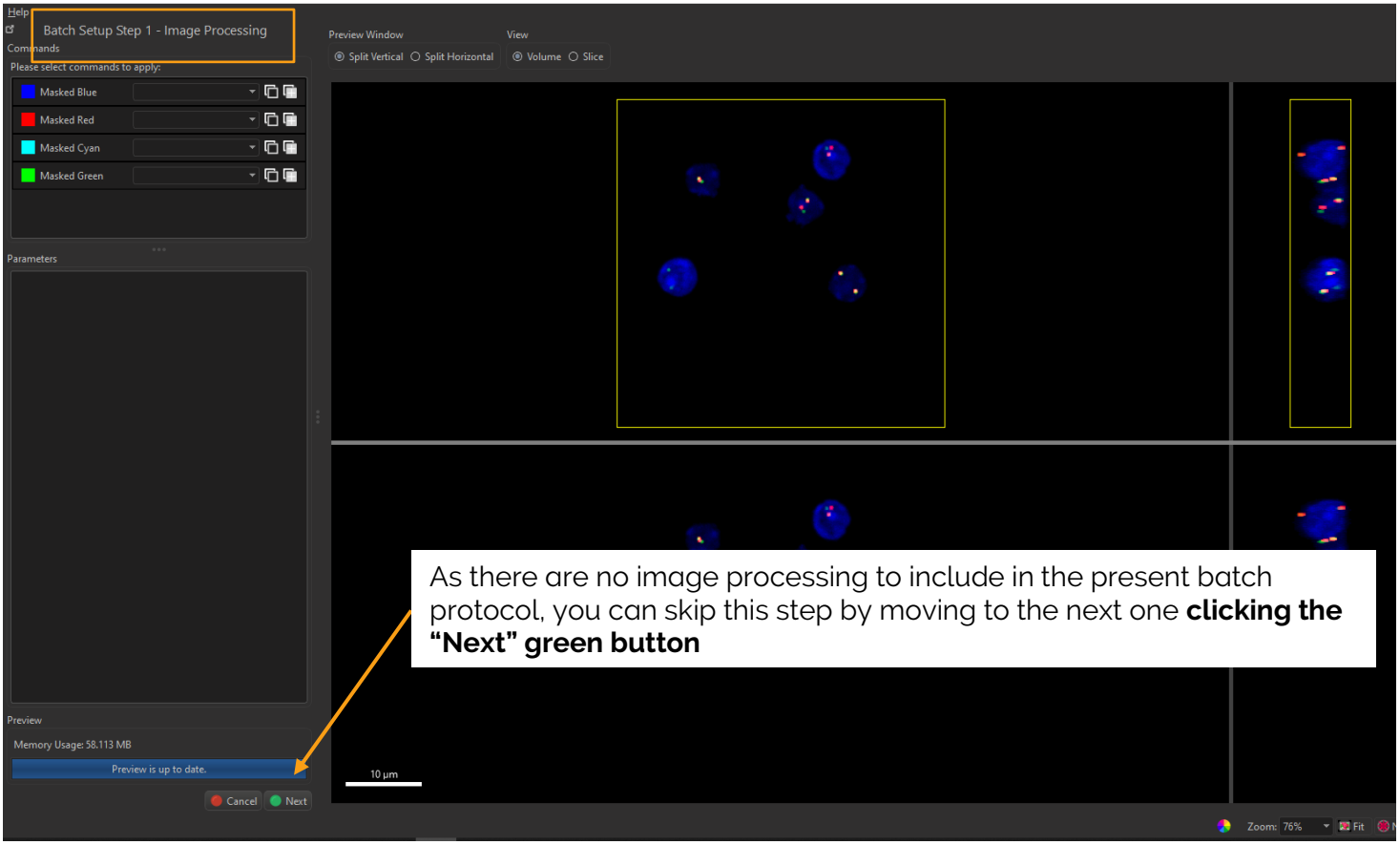



To explore all steps of the batch protocol we need to use an image as a template. Therefore double click on the Experiment1 folder to open it:





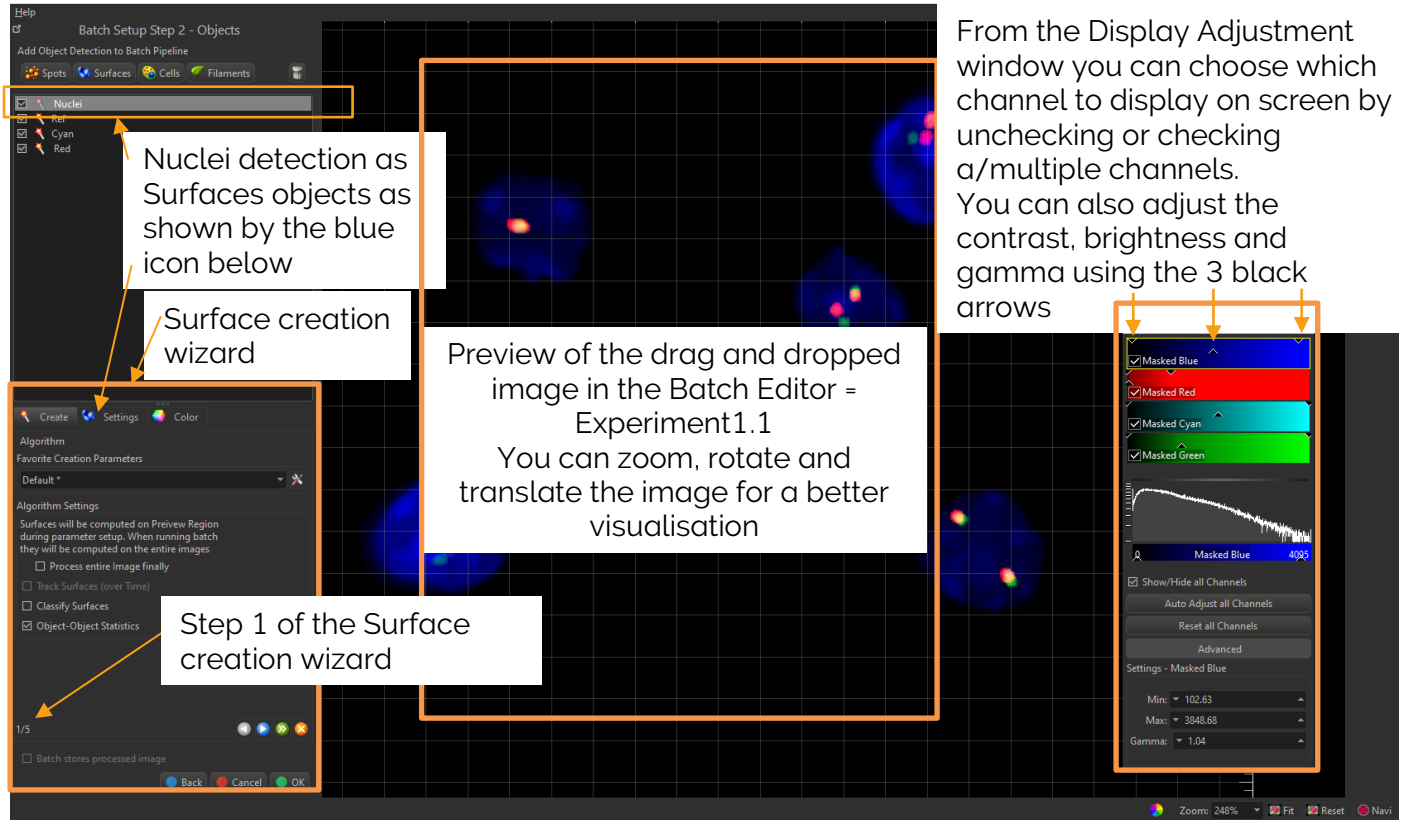

This opens the batch set-up editor interface at step 1 of the protocol creation wizard. This steps can enable image processing such as deconvolution, inversion of a channel, Gaussian filters, background substraction etc. We will not perform any image processing in the present exercise so you can skip this step as follow:



In Step 2 of the Batch Set-up, we can see that 4 different objects will be created:

- Nuclei
- Ref
- Cyan
- Red

By highlighting any object name, its wizard of creation appears at the bottom left letting you know also its object type: Spots, Surfaces, Cell or Filament.
If one needs to create an extra object, he can click on the top of the list icons (Spots or Surfaces or Cell or Filament). There is no need to create new objects in this exercise.

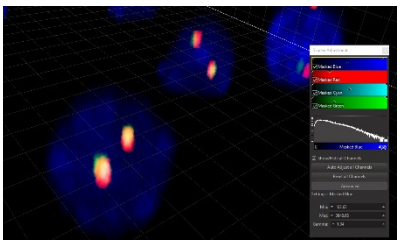



From the Display Adjustment window you can choose which channel to display on screen by unchecking or checking a/multiple channels.
You can also adjust the contrast, brightness and gamma using the 3 black arrows

! If you don't see the "Display Adjustment" window hit on the keyboard: Ctrl + D

Image description:

- Ch1: Blue nuclei
- Ch2: Red alleles
- Ch3: Cyan alleles
- Ch4: Green alleles



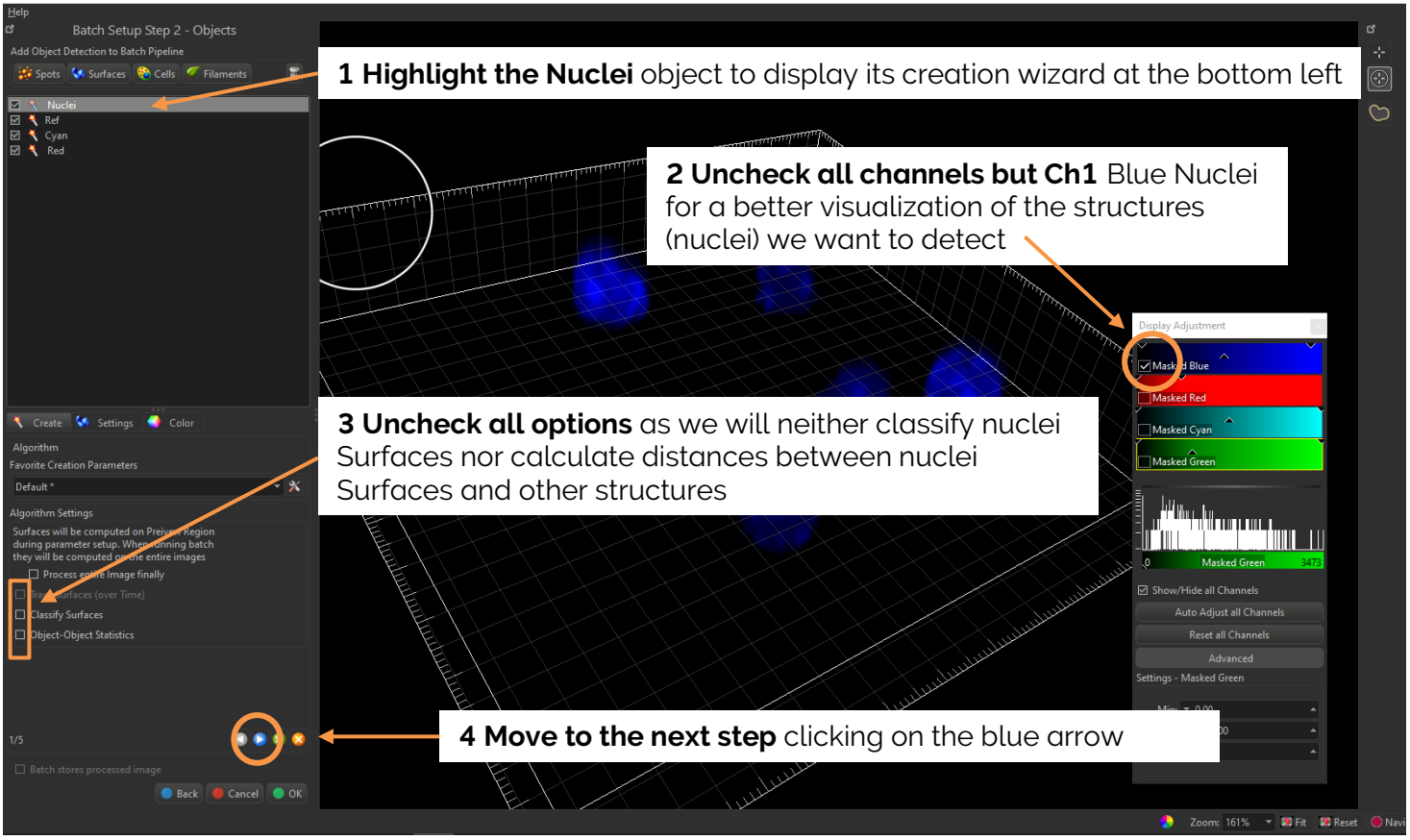
- We will detect all nuclei per image as Surfaces objects to get their counts, volumetric and morphological descriptions. This can be useful if one needs to normalise the batch results at the end. This normalisation will not be shown in the present exercise.

- We need to detect the Red and Cyan alleles (as Spots) then measure the distance between them to see if they colocalize (using the Spots-Spots Shortest Distance statistic), then classify each Red and Cyan allele as a Colocalizing allele or Not Colocalizing.

- We will not use Ch4 green in this exercise.

## Set-up Nuclei detection as Surfaces for Batch

The aim of the exercise is to find colocalizing alleles, however if one would like to normalize results per image using the number of cells/nuclei, we need to also detect nuclei.

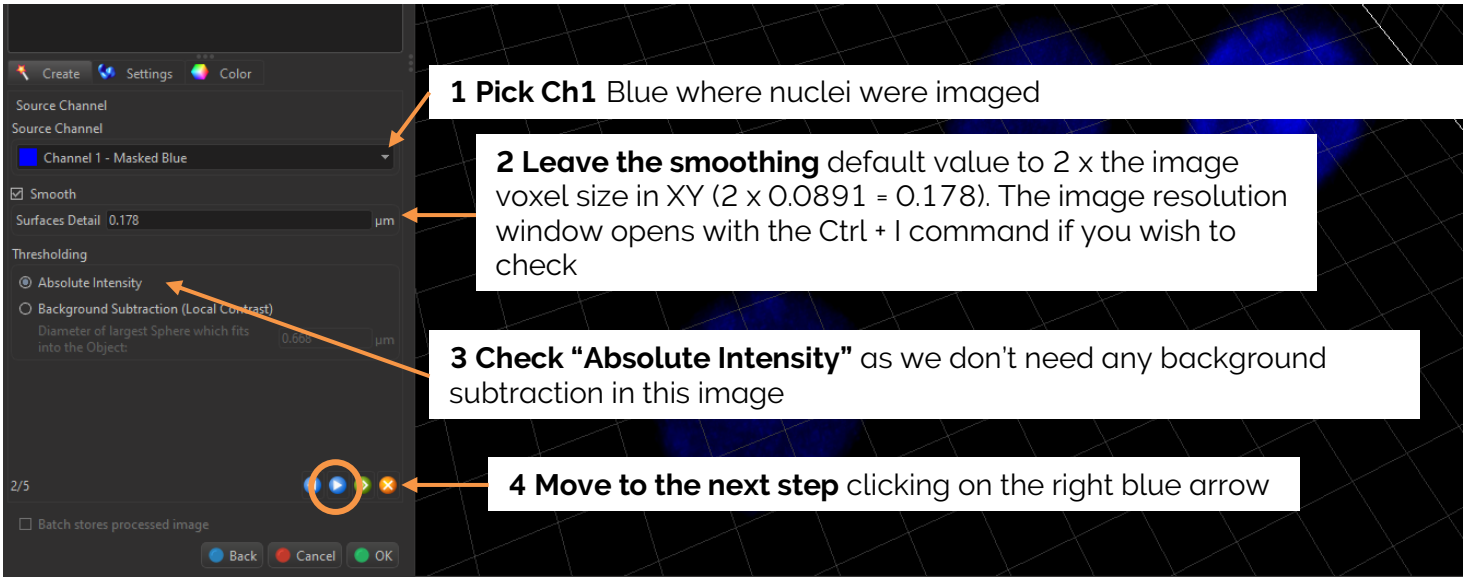



Option explanations:

- **Process entire Image finally**: if checked, the full image will be processed at the end of the wizard set-up. During the wizard parameters set-up, only a region of interest is display in the 3D scene.
- .**Classify Surfaces:** if checked, once Surfaces are detected, it enables automatic Surfaces Classification using either 1D or 2D filters or Machine Learning.
- **Object-Object statistics:** if checked, it enables the calculation of distances between objects (Spots-Surfaces and Surfaces-Surfaces)

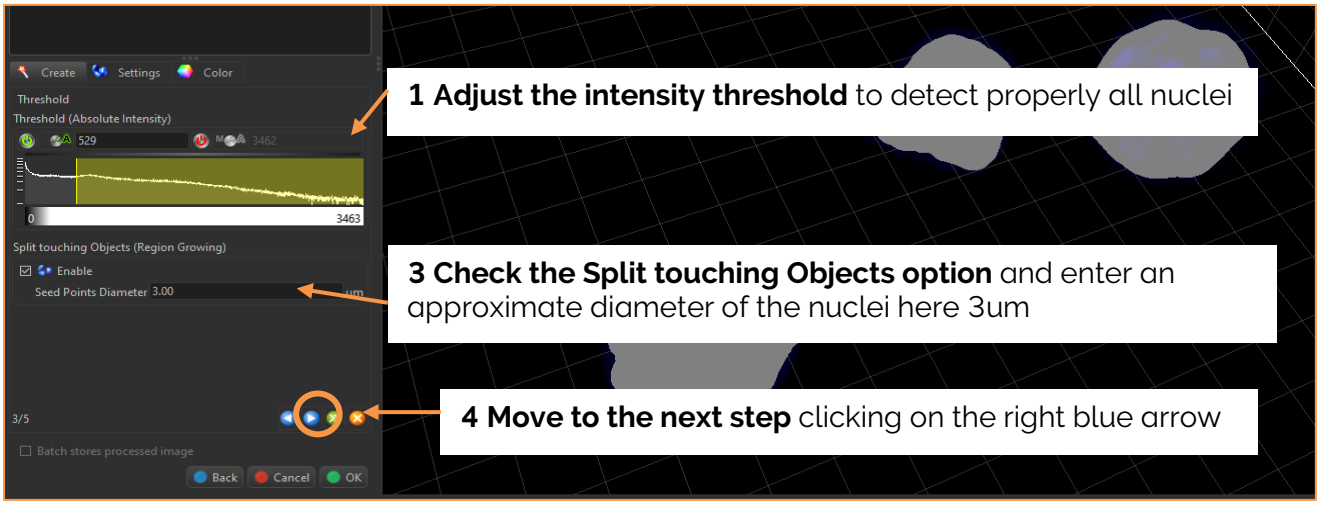
➢ **Blue arrow:** to move from one step to the next one or previous one

➢ **Green arrow:** to finish the wizard of creation automatically using already entered parameters.



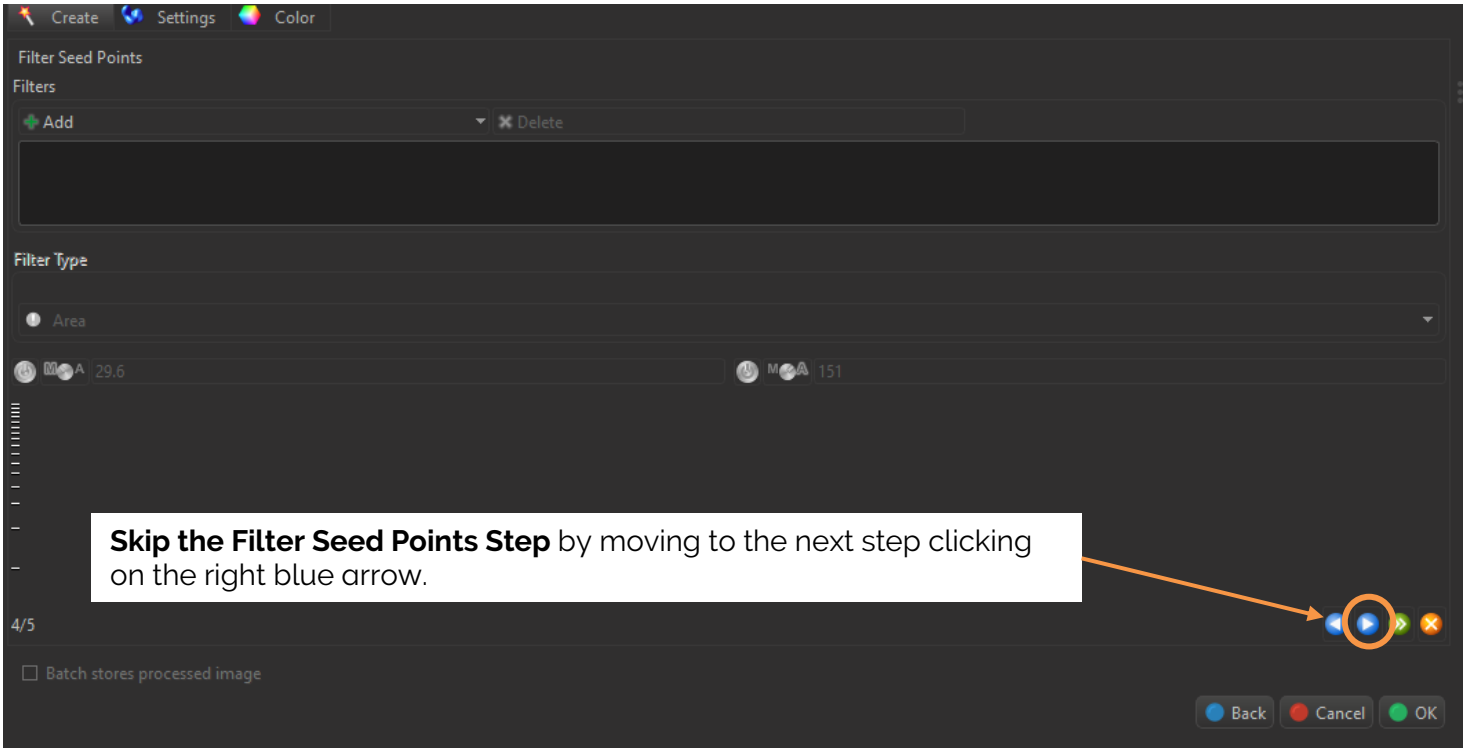

Option explanations:

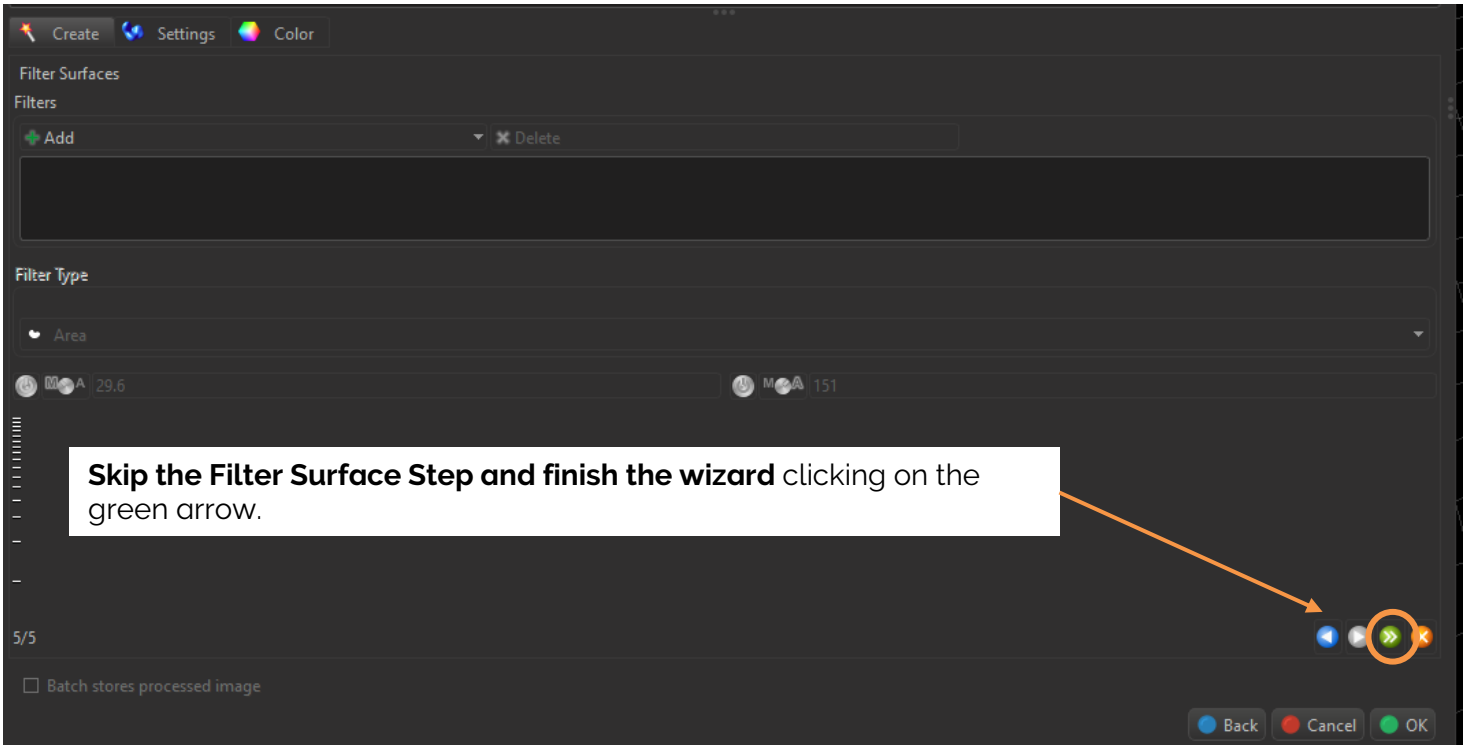
- **Smooth:** sets up the smoothness of the resulting area. Smoothing applies a Gaussian filter to the data set. If the option Smooth is selected, then the Surfaces Detailed Level value should be determined.
- **Background Subtraction:** if checked, it applies a Gaussian filter to estimate the background intensity value of each voxel. This variable background is then subtracted from every voxel in the image; Baseline Subtraction is performed. The width of the Gaussian filter is determined by
- the diameter of the largest sphere that fits into the Object from the intensity of the original channel.



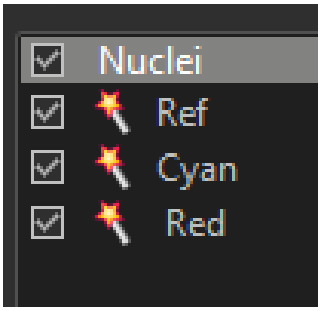

Option explanations:

- **Threshold**: Objects with values below the Lower Threshold and objects with values exceeding the Higher Threshold will be excluded and only those objects with quality inside the interval defined by the two threshold values are considered.
- **The Green A stands for Automatic thresholding**: the initial threshold value is automatically computed using an algorithm based on [T.W. Ridler and S. Calvard, "Picture thresholding using an iterative selection method", IEEE Trans. System, Man and Cybernetics, SMC-8 (1978) 630-632]. If you do a Manual thresholding, then you'll see a green M
- **Split touching Objects (Region Growing):** This option supports the separation of two or more objects that are identified as one. The region method for segmentation in Imaris starts with defining seed points in each peak center. Then a region growing process will run around each seed point where the region grows until a border stops the growing. The threshold you set above is the border of the region growing



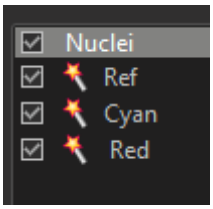




The Surface wizard of creation to detect Nuclei is ready. The small magic wand icon in front of the Nuclei Surfaces object disappears.



## Set-up Reference Red alleles detection as Spots for Batch

We are interested in the colocalization between the Red and Cyan alleles. However, you'll notice that we have 3 Spots objects and the first one is named "Ref".




Indeed, when Imaris will generate the first Spots object "Ref" (for reference) there will be no other Spots object to calculate any inter-object distances at this stage so the Object-Object Statistics option will not have any effect just yet

For the Object-Object Statistics option to work, Imaris needs a second Spots object to be created, here the Cyan alleles Spots, so that it outputs the distances between all Cyan and Red Spots "Ref".

In the Cyan Spots alleles statistics we'll find::

- The measurement: Shortest distance from Cyan alleles Spots to Red alleles Spots 'Ref"

- And thanks to the classification step:

    - the number of Cyan alleles colocalizing with Red alleles that is to say that are below a user defined distance threshold from the Red allele Spots

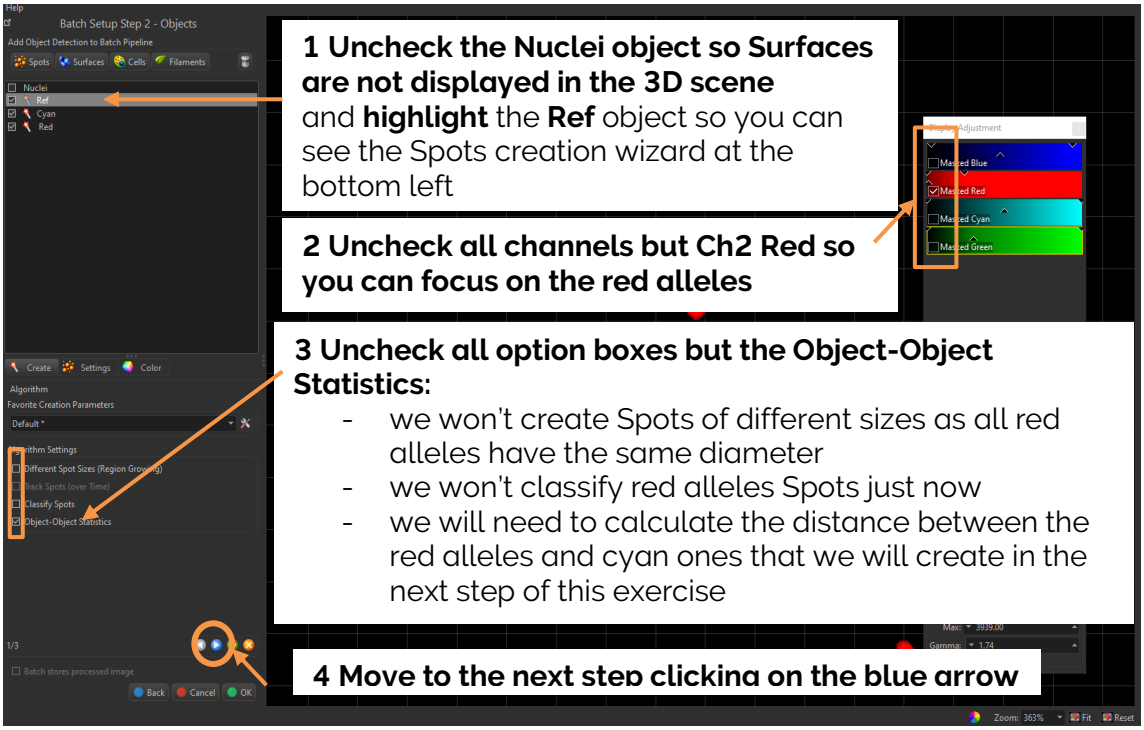
    - the number of Cyan alleles NOT colocalizing with Red alleles that is to say that are above a user defined distance threshold from the Red allele Spots

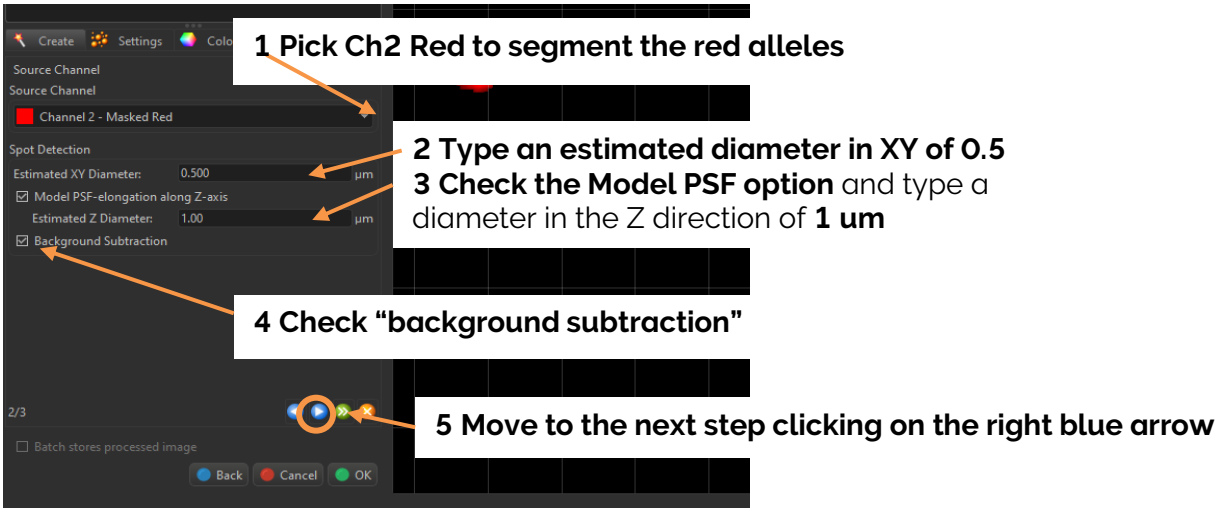
For this calculation to happen, the option Object-Object Statistics has to be enabled (checked) in both Red "Ref" and Cyan Spots creation wizards.

Further, we'll need to re-create Red allele Spots named "Red" so that the Shortest distance from Red to Cyan will be also available in the Red alleles Spots statistics:

1- By identifying the number of colocalized Spots for both Red and Cyan, we can calculate the stoichiometry of these two alleles. In this experiment, its 1:1, but you may see different stoichiometry in other experiments

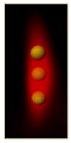
2- We also would like to get the number of Red allele Spots that do NOT colocalize with Cyan allele Spots. So we need to also classify Red allele Spots in 2 classes: the one colocalizing with Cyan alleles (below a user defined distance from Cyan Spots) and the NOT colocalizing with Cyan (above below a user defined distance from Cyan Spots)



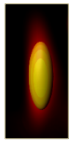




- **Source Channel:** Select the channel containing the structures you want to detect as Spots.
- **Estimated Diameter;** Type in an estimation of the diameter of your structures. Objects with size smaller than the specified Estimated Diameter are not detected.
- **Model PSF-elongation along Z-axis:** This is to create elliptical Spots in opposition to spherical Spots which is the default. This parameter specifies the dimension of the Spots object in the Z direction (usually 2 x XY diameter). This is useful for images where the signal is largely stretched in the Z direction like in Wide Field images not deconvolved. Z elongation is most prominent when working with objects close to diffraction limit and happens to most common types of microscopes (widefield, confocal, etc.) It can correct for artefacts as shown below:

Volume Rendered Object

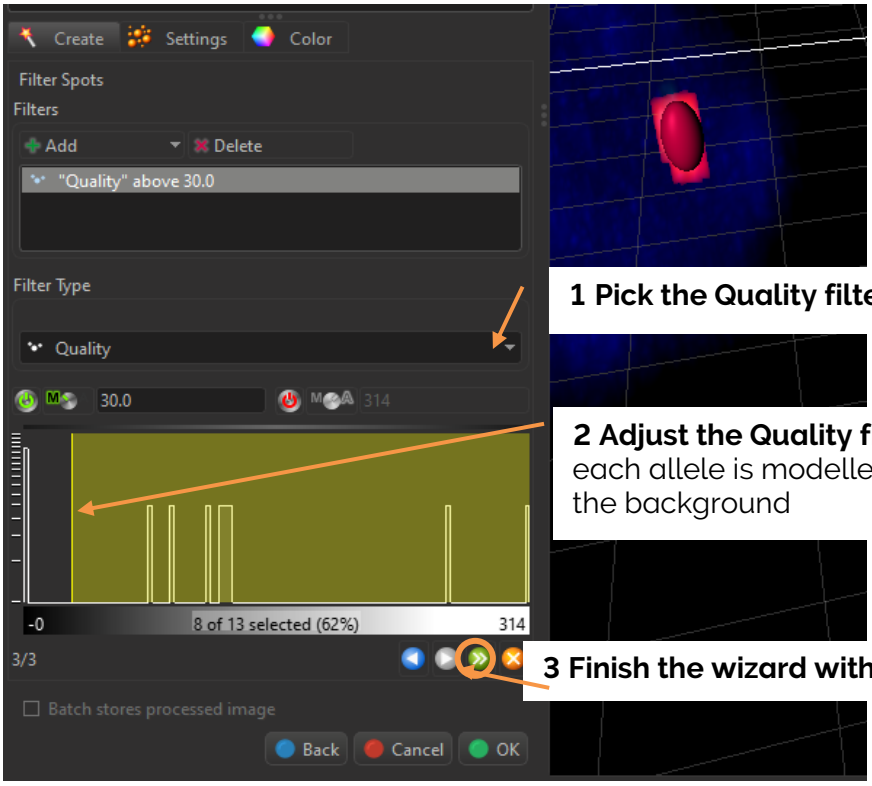


Spherical Spots (artifact)



Ellipsoid Spots (correct)

- **Background Subtraction:** the background will be removed prior to Spots' detection. Technically background subtraction smoothes the image. The intensity is the one of a Gaussian filtered channel (Gaussian filtered by 3/4) minus the intensity of the original channel Gaussian filtered by 8/9 of sphere radius.

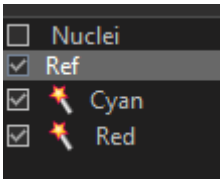



**1 Pick the Quality filter**

**2 Adjust the Quality filter** looking at the 3D scene so that each allele is modelled by a Spot and no extra Spots are in the background

**3 Finish the wizard with green arrow**

The Spots wizard of creation to detect red alleles as "Ref" is ready. The small magic wand icon in front of the Ref object disappears.




Option explanations:

**Histogram**: In the histogram the regions above and below the threshold values are displayed in white, while region within threshold is displayed in yellow

- The green A stands for Automatic threshold
- The green M stands for Manual threshold
- Left click to move the lower threshold
- Right click to move the higher threshold
- Scroll to zoom in the histogram
  The histogram has a zoom bar at the top:
  - Mouse wheel allows to zoom in and out.
  - Holding right-click on the zoom bar allows to pan.
  - Double right-click resets the zoom.
  -

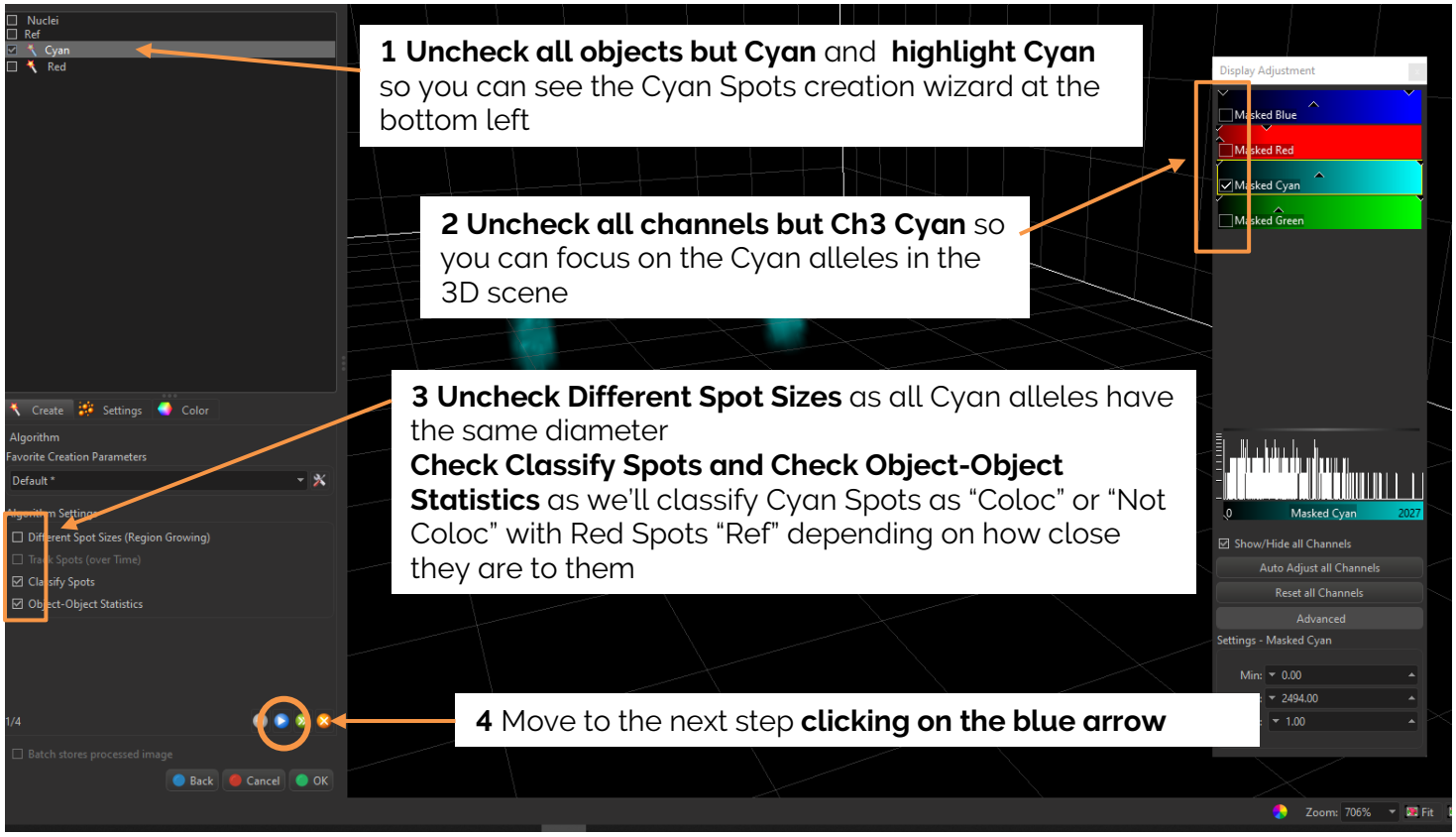
**Spots filtering** is incorporated into the creation process. Filters allow the removal of objects that would interfere with interpretation of results.

**Quality**: The 'Quality' is the intensity at the centre of the spot in the channel the Spots was detected.

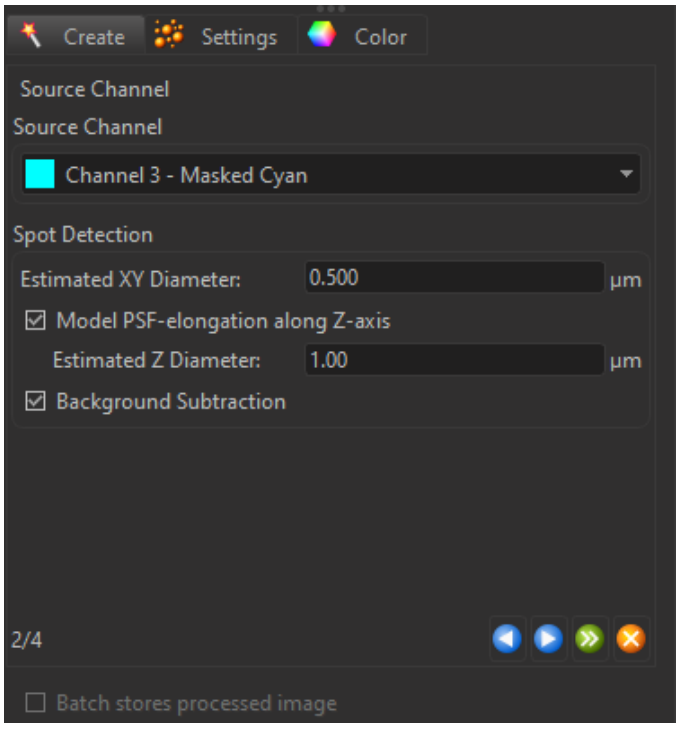
- If 'Background Subtraction' was not checked this is the intensity of the channel Gaussian filtered by 3/4 of spot radius.

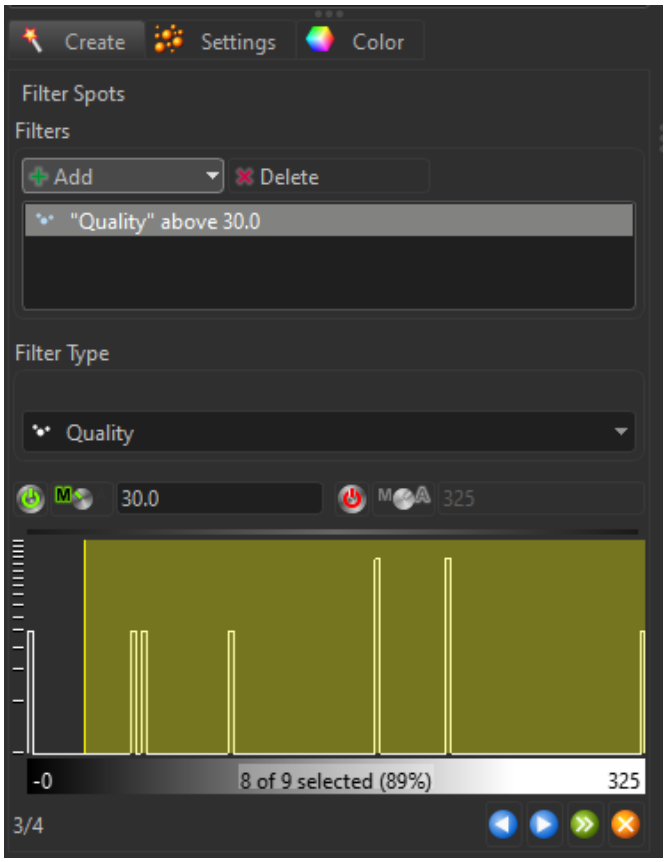
- If 'Background Subtraction' was active the intensity is the one of a Gaussian filtered channel from above minus the intensity of the original channel Gaussian filtered by 8/9 of spot radius.

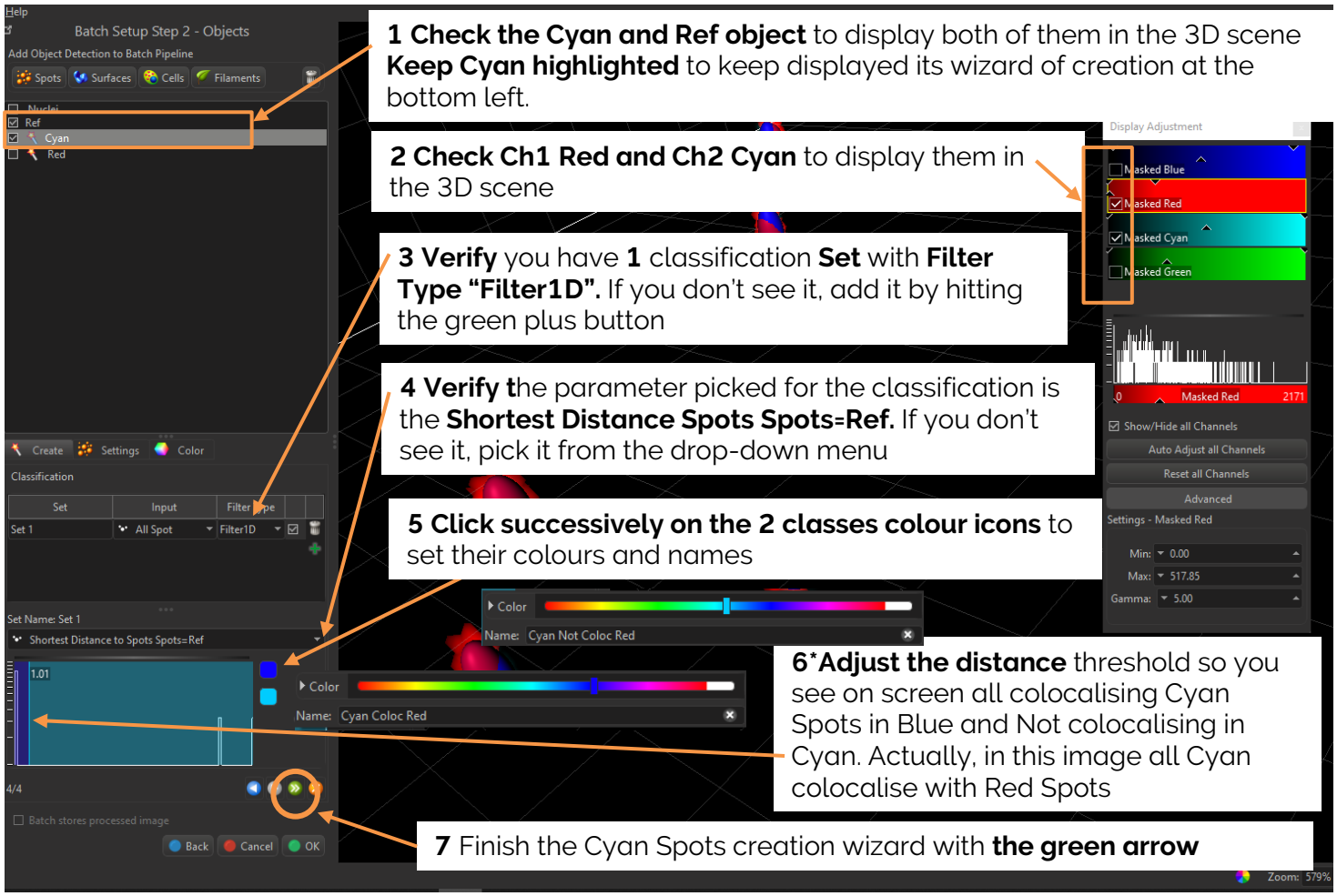
## Set-up Cyan alleles detection as Spots for Batch




Fill in the next steps as follow:

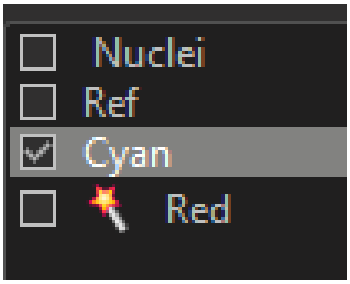





*Note: the distance measurement for Spots is from the center of the Spots, and the threshold we set (1 um, 2x Spot diameter) means the two Spots are at least "touching"

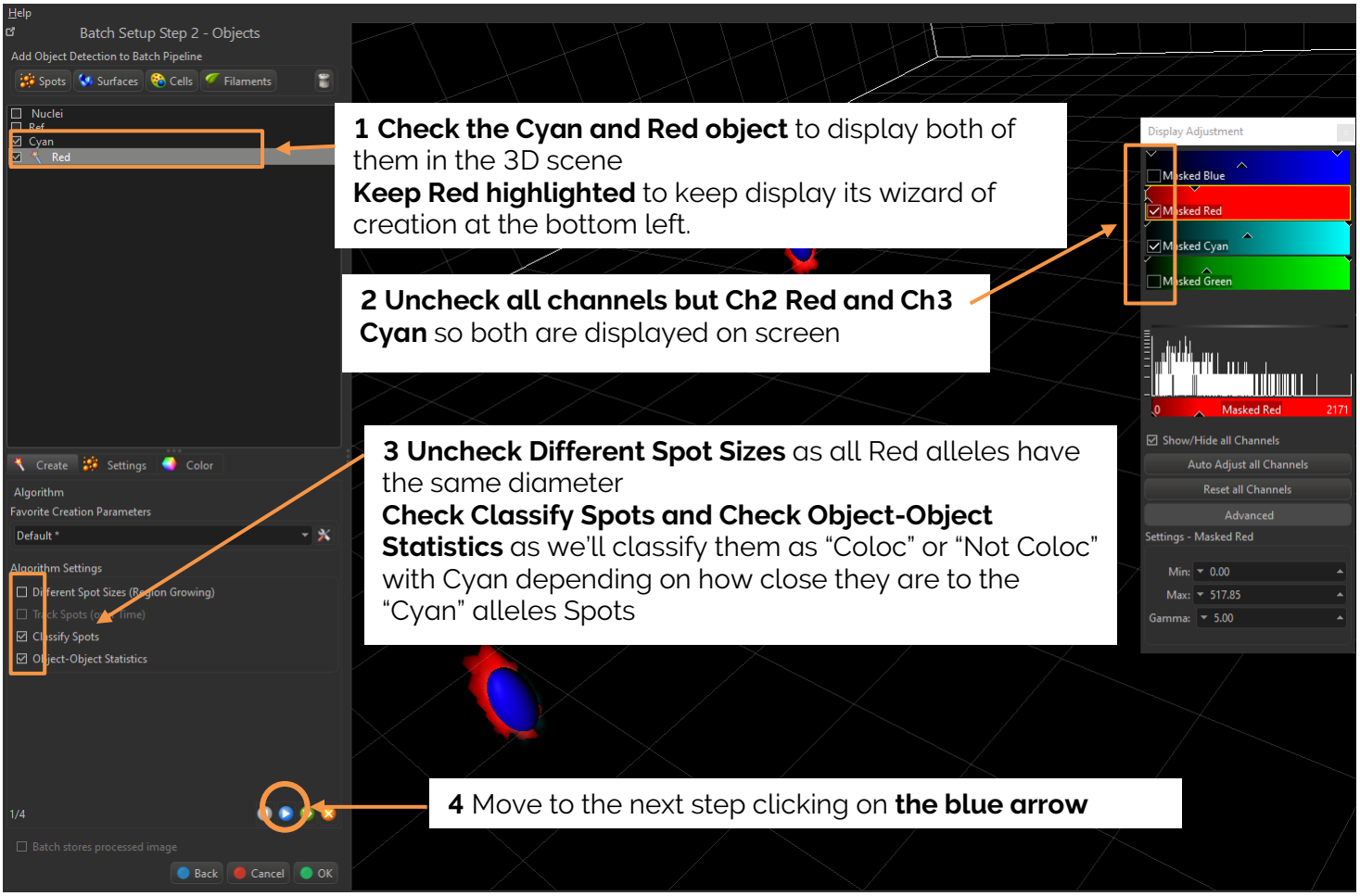
The Spots wizard of creation to detect Cyan alleles as "Cyan" Spots is ready. The small magic wand icon in front of the "Cyan" object disappears.



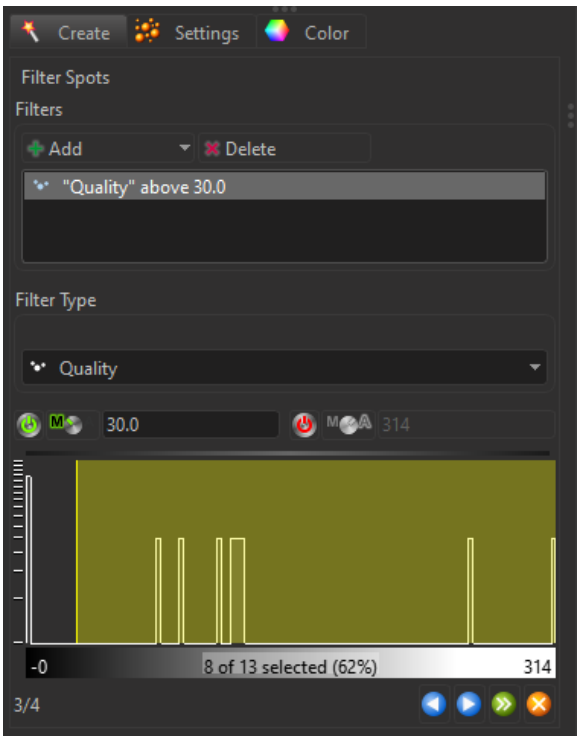

## Set-up Red alleles detection as Spots for Batch

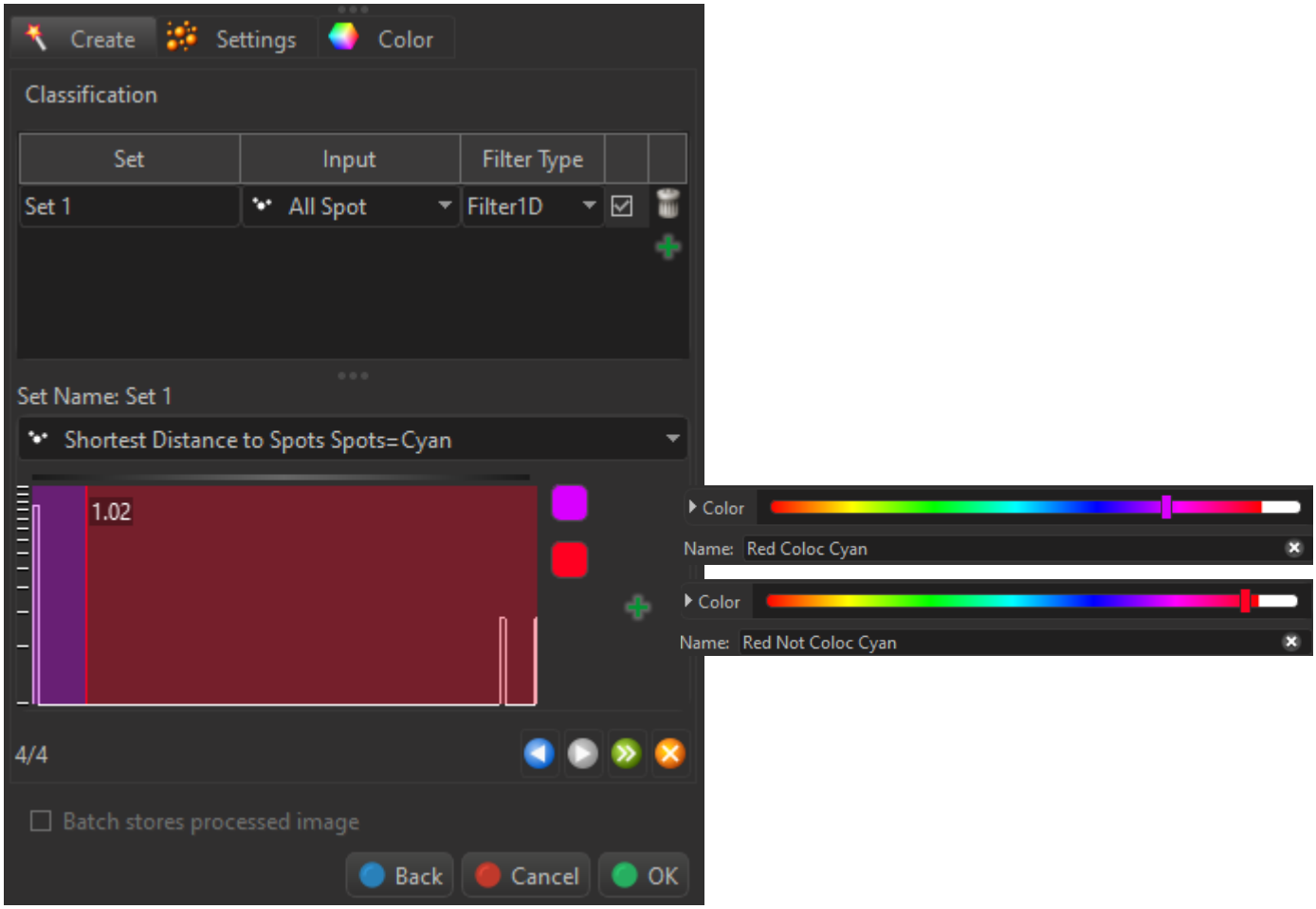
As mentioned before, we need to re-create Red allele Spots named "Red" so that the Shortest distance from Red to Cyan will be also available in the Red alleles Spots statistics. This way, we'll get the number of Red alleles Spots that colocalize with Cyan alleles, and the number of Red allele Spots that do NOT colocalize with Cyan allele Spots.

Proceed as follow similarly to the previous step:

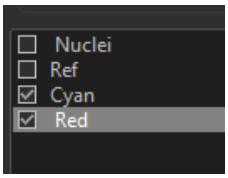

Help
Batch Setup Step 2 - Objects
Add Object Detection to Batch Pipeline
Spots
Surfaces
Cells
Filaments
Nuclei
Ref
Cyan
Red
Create
Settings
Color
Algorithm
Favorite Creation Parameters
Default *
Algorithm Settings
Different Spot Sizes (Region Growing)
Track Spots (over Time)
Classify Spots
Object-Object Statistics
1/4
Batch stores processed image
Back
Cancel
OK
1 Check the Cyan and Red object to display both of them in the 3D scene
Keep Red highlighted to keep display its wizard of creation at the bottom left.
2 Uncheck all channels but Ch2 Red and Ch3 Cyan so both are displayed on screen
3 Uncheck Different Spot Sizes as all Red alleles have the same diameter
Check Classify Spots and Check Object-Object Statistics as we'll classify them as "Coloc" or "Not Coloc" with Cyan depending on how close they are to the "Cyan" alleles Spots
4 Move to the next step clicking on the blue arrow
Display Adjustment
Masked Blue
Masked Red
Masked Cyan
Masked Green
Masked Red
2171
Show/Hide all Channels
Auto Adjust all Channels
Reset all Channels
Advanced
Settings - Masked Red
Min: 0.00
Max: 517.85
Gamma: 5.00


Create
Settings
Color
Source Channel
Source Channel
Channel 2 - Masked Red
Spot Detection
Estimated XY Diameter:
0.500
µm
Model PSF-elongation along Z-axis
Estimated Z Diameter:
1.00
µm
Background Subtraction
2/4

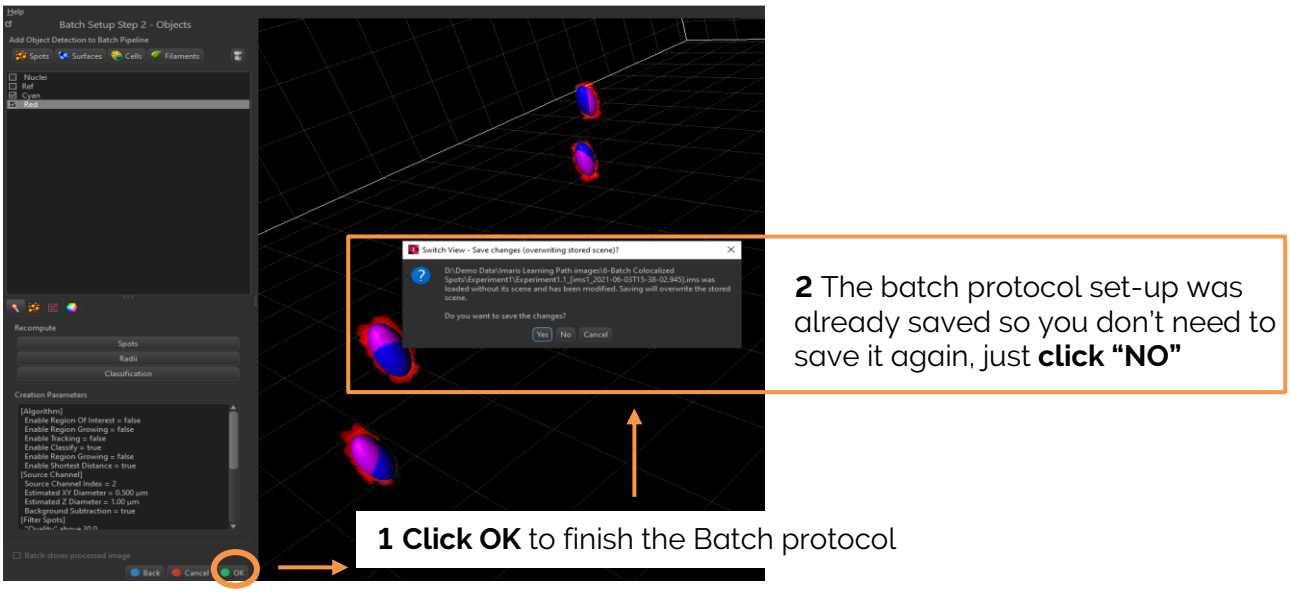

Create
Settings
Color
Filter Spots
Filters
Add
Delete
"Quality" above 30.0
Filter Type
Quality
30.0
314
-0
8 of 13 selected (62%)
314
3/4



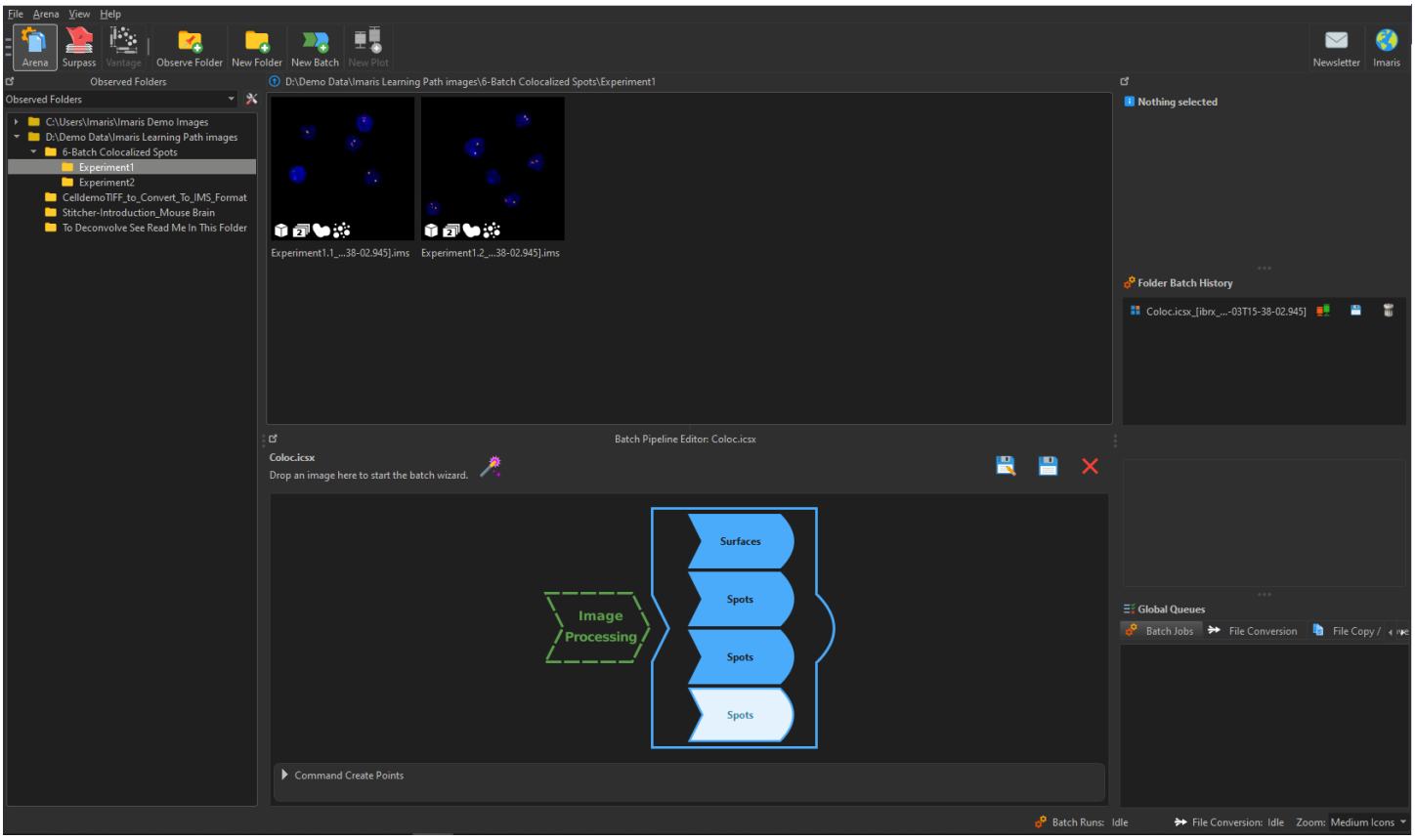

The Spots wizard of creation to detect Red alleles as "Red" Spots is ready. The small magic wand icon in front of the "Red" object disappears.

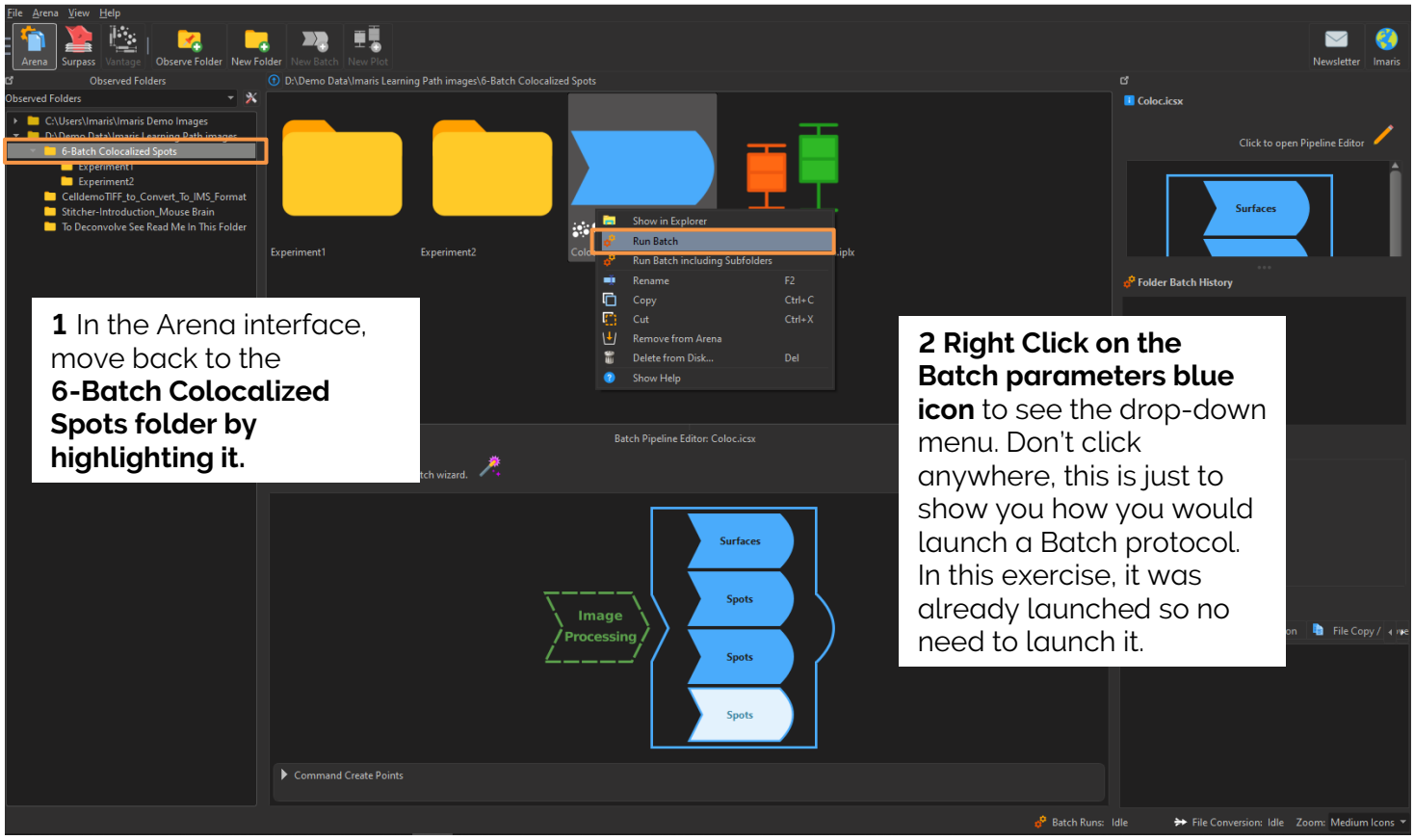



## Batch process launch



The Batch Editor will close:

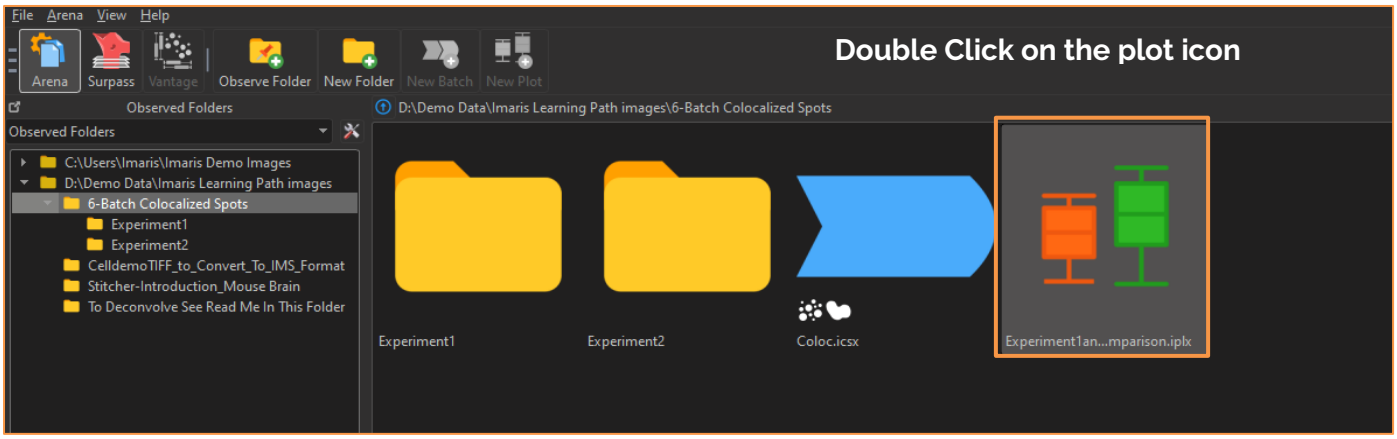


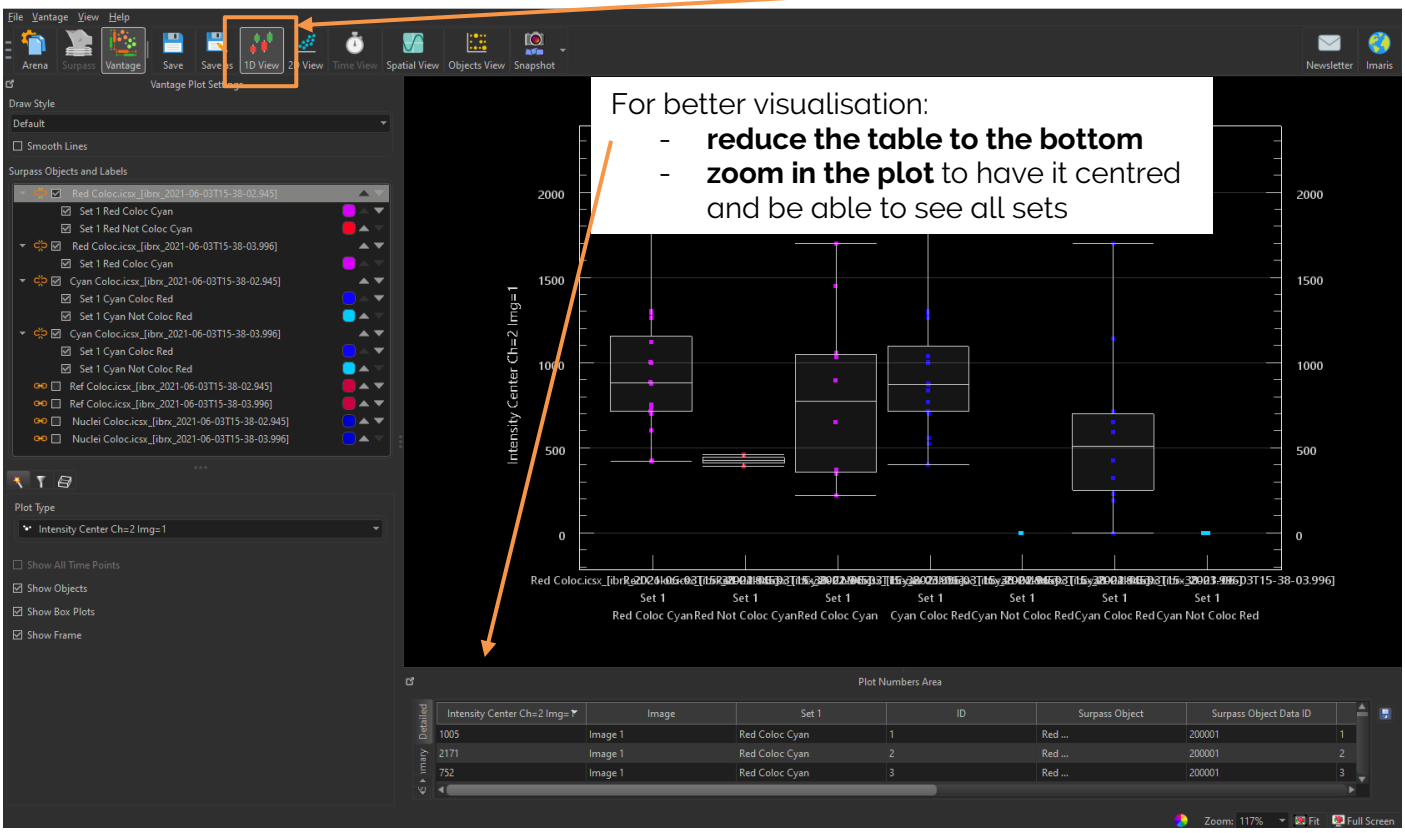

**1** In the Arena interface, move back to the **6-Batch Colocalized Spots folder by highlighting it.**

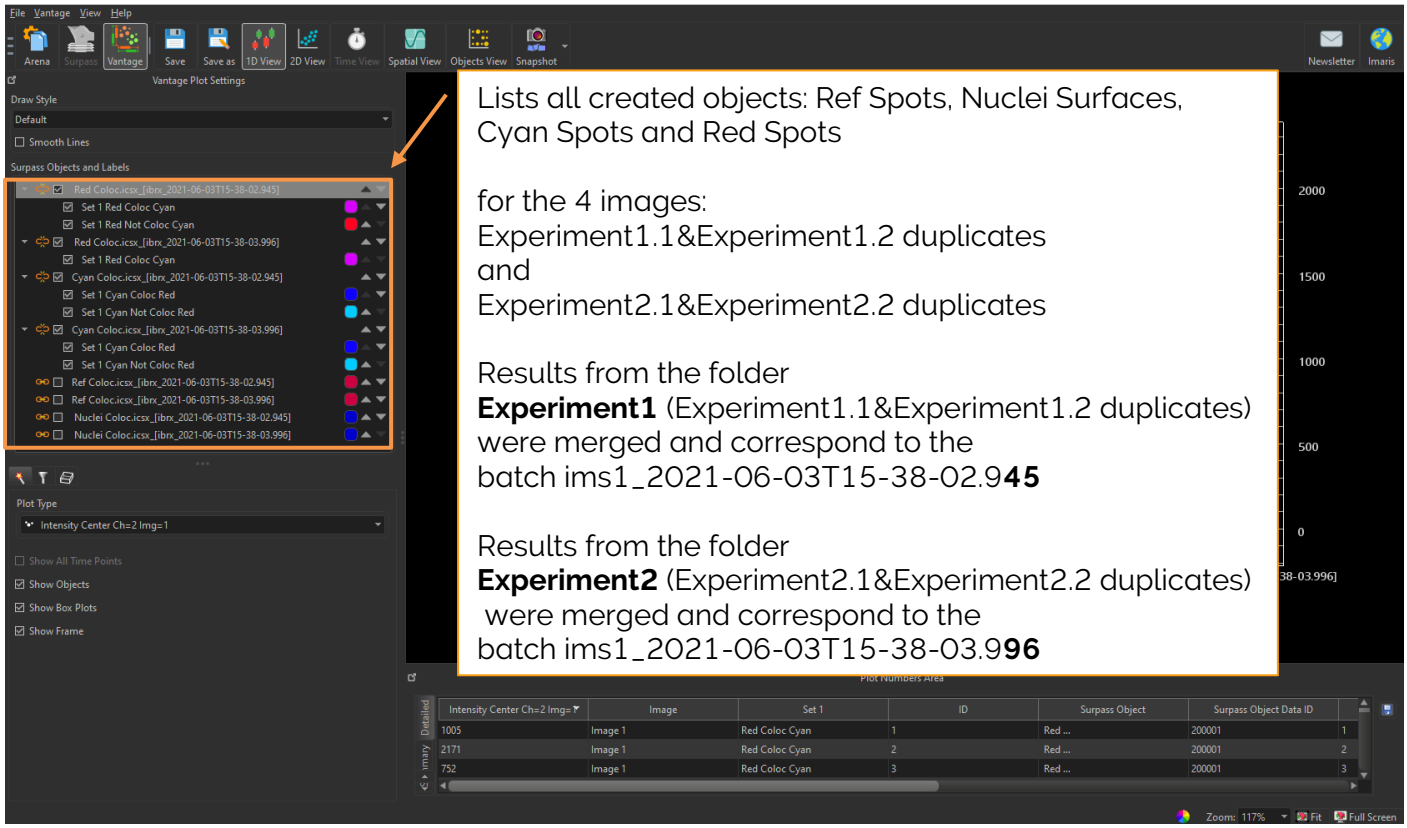
**2 Right Click on the Batch parameters blue icon** to see the drop-down menu. Don't click anywhere, this is just to show you how you would launch a Batch protocol. In this exercise, it was already launched so no need to launch it.

## 1D comparison plot of the 2 experiments

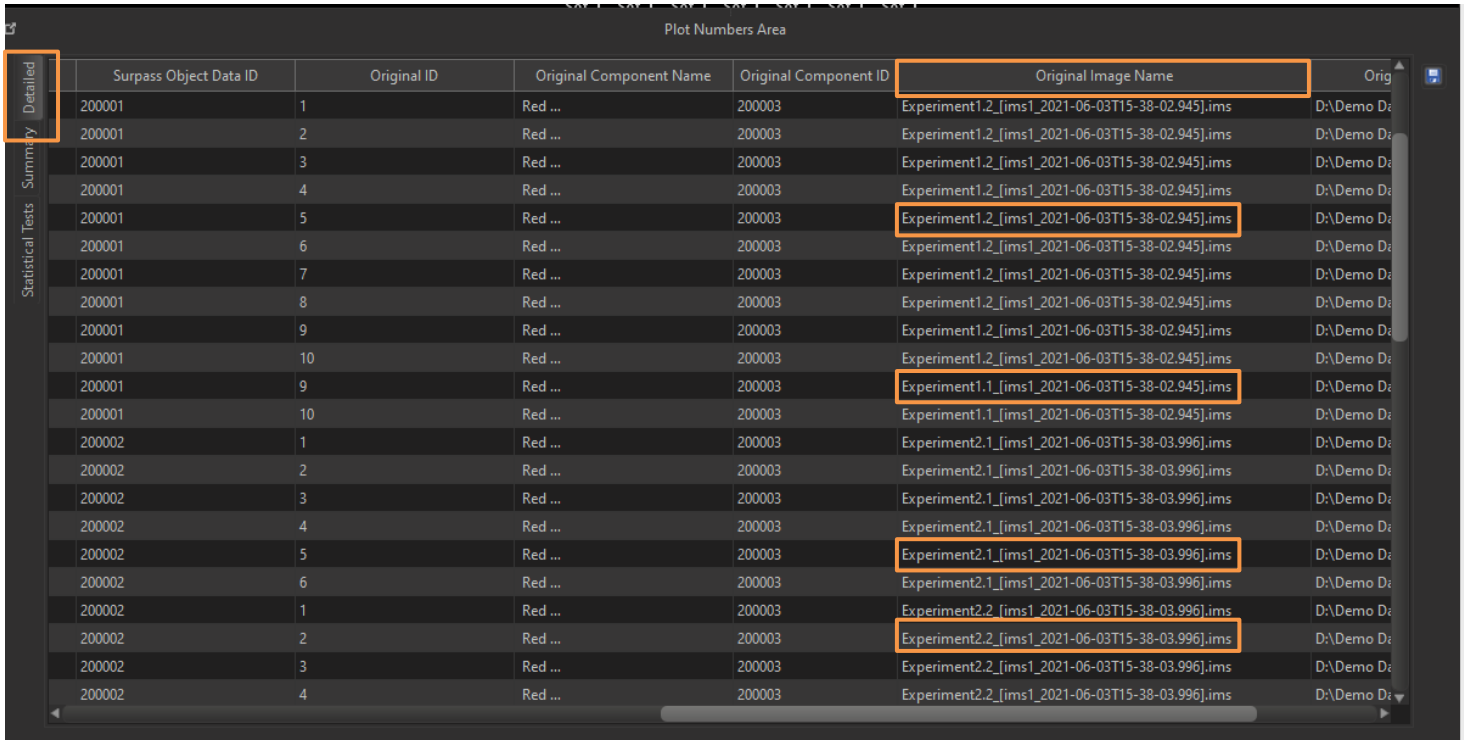



If you don't see this plots in the Vantage interface, please make sure you are in the 1D View

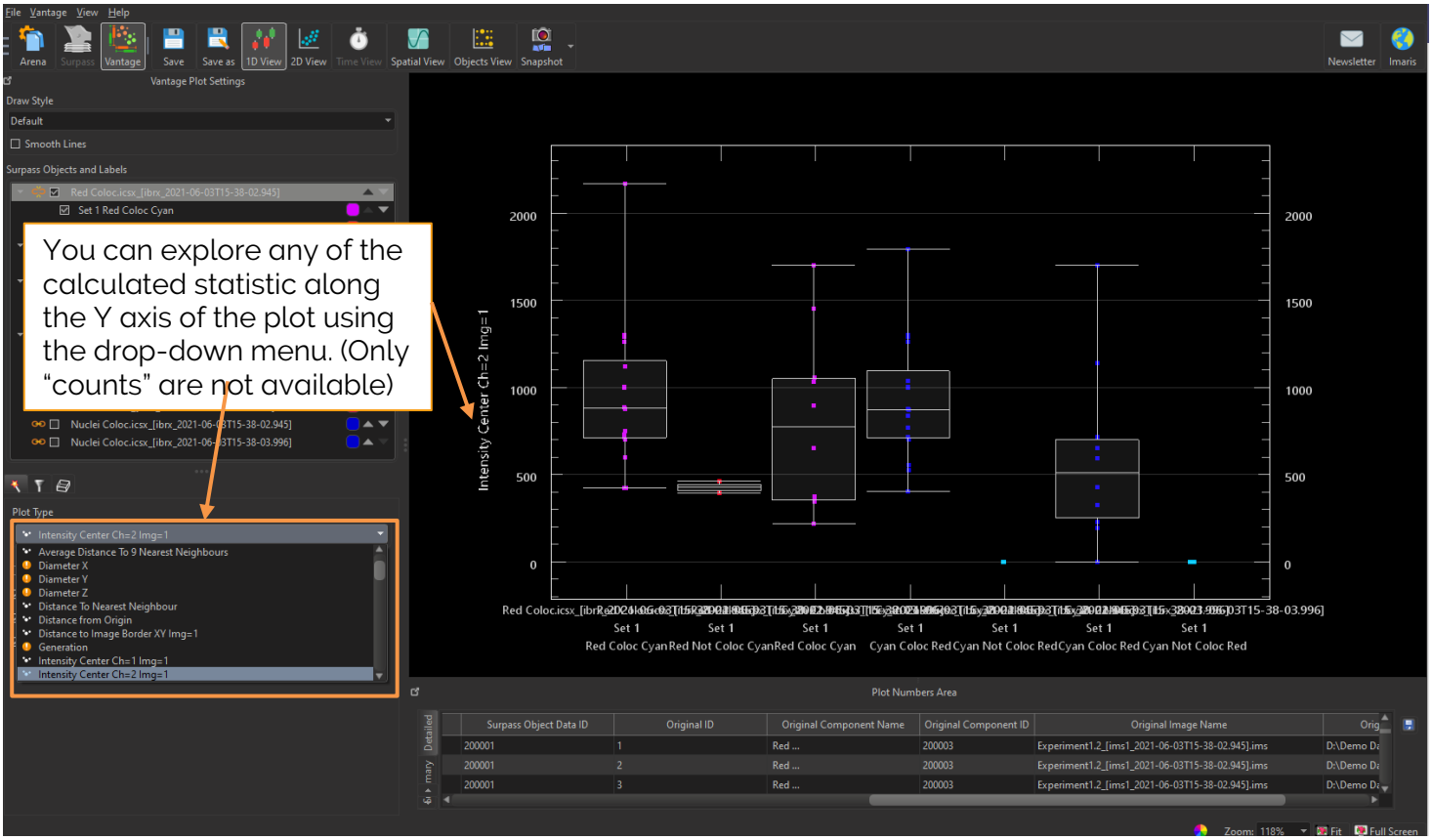


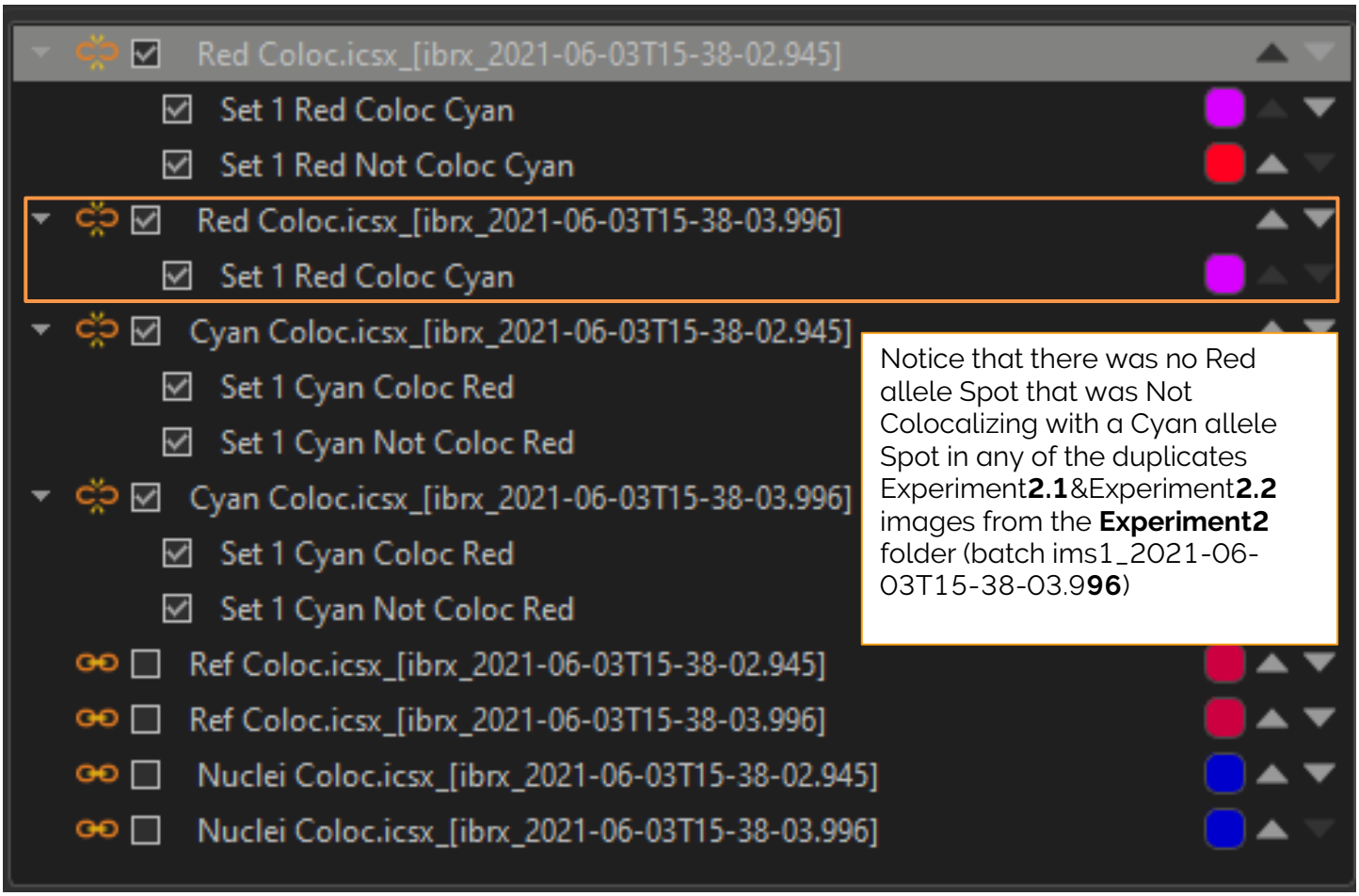



To know which batch number (ims1_2021-06-03T15-38-02.945 **or** ims1_2021-06-03T15-38-03.996) corresponds to which folder (Experiment1 **or** Experiment2) you can develop the table of results under the "Detailed" tab and focus on the "Original Image Name" column as follow:

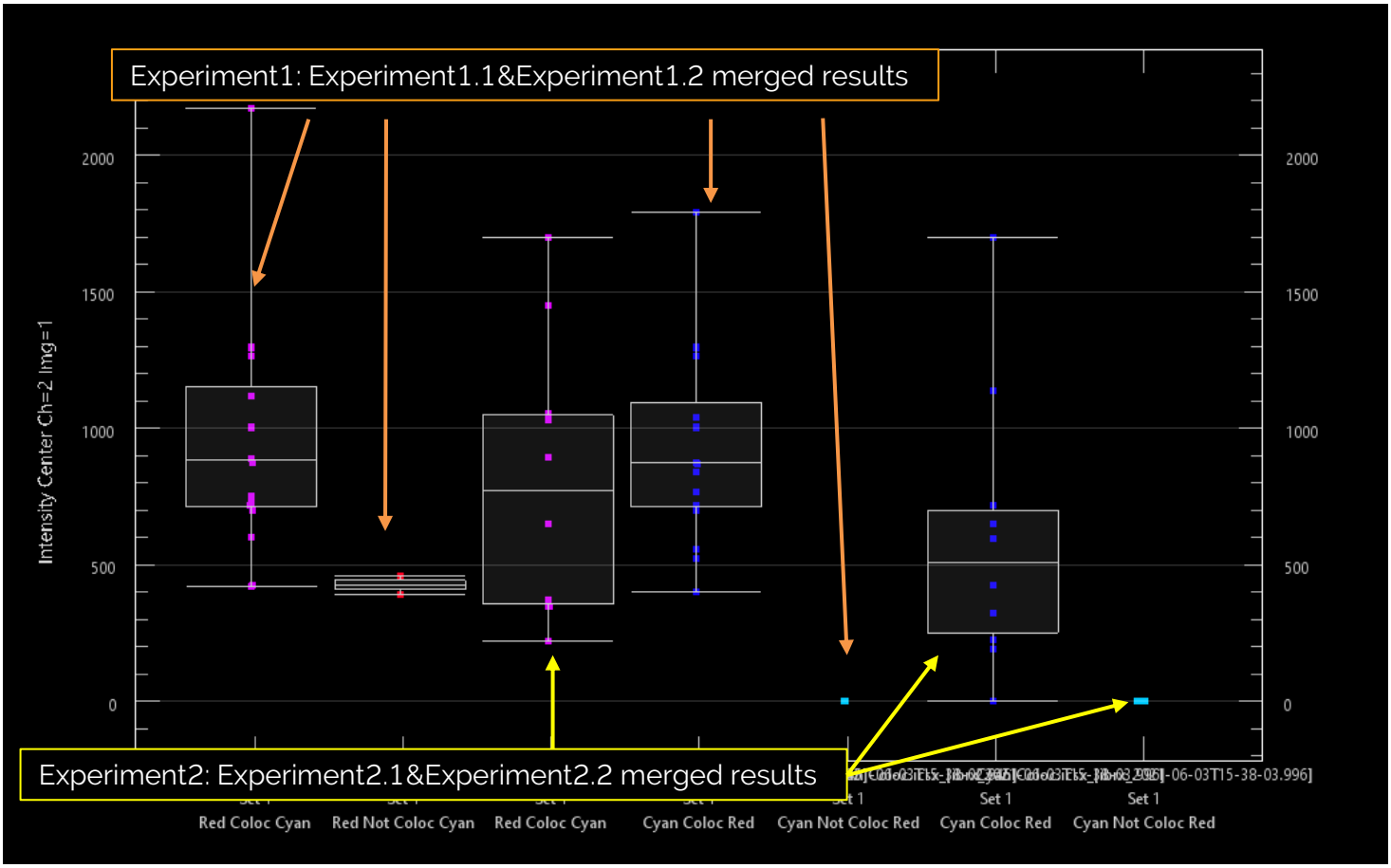



| Surpass Object Data ID | Original ID | Original Component Name | Original Component ID | Original Image Name | Orig |
|---|---|---|---|---|---|
| 200001 | 1 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 2 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 3 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 4 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 5 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 6 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 7 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 8 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 9 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 10 | Red ... | 200003 | Experiment1.2_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 9 | Red ... | 200003 | Experiment1.1_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200001 | 10 | Red ... | 200003 | Experiment1.1_[ims1_2021-06-03T15-38-02.945].ims | D:\Demo Da |
| 200002 | 1 | Red ... | 200003 | Experiment2.1_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 2 | Red ... | 200003 | Experiment2.1_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 3 | Red ... | 200003 | Experiment2.1_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 4 | Red ... | 200003 | Experiment2.1_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 5 | Red ... | 200003 | Experiment2.1_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 6 | Red ... | 200003 | Experiment2.1_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 1 | Red ... | 200003 | Experiment2.2_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 2 | Red ... | 200003 | Experiment2.2_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 3 | Red ... | 200003 | Experiment2.2_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |
| 200002 | 4 | Red ... | 200003 | Experiment2.2_[ims1_2021-06-03T15-38-03.996].ims | D:\Demo Da |



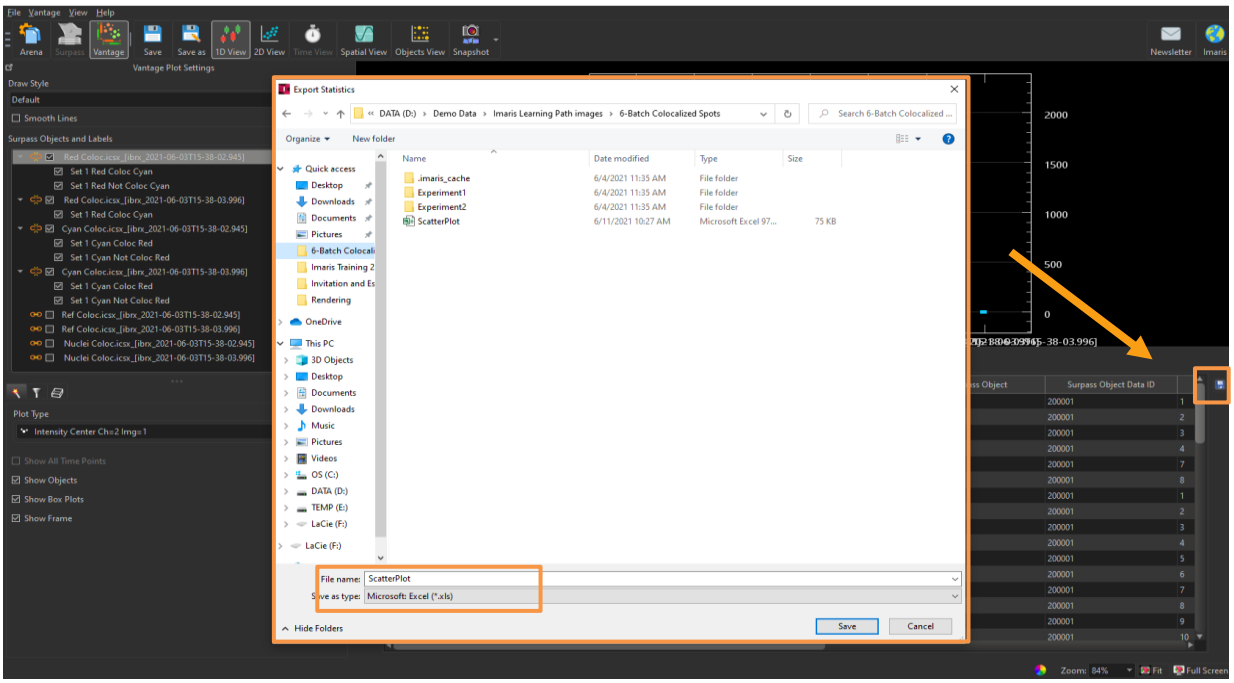




Notice that there was no Red allele Spot that was Not Colocalizing with a Cyan allele Spot in any of the duplicates Experiment**2.1**&Experiment**2.2** images from the **Experiment2** folder (batch ims1_2021-06-03T15-38-03.9**96**)



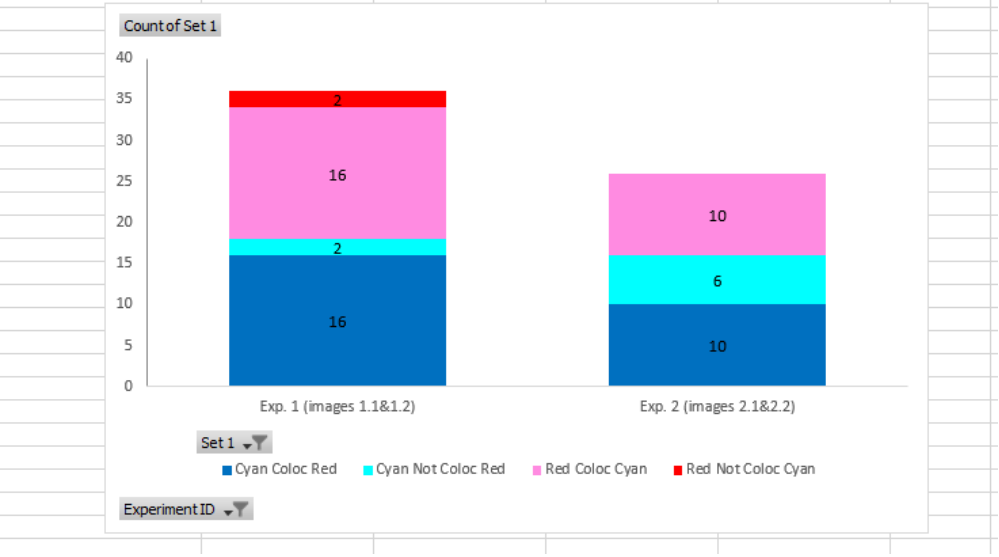

## Export statistics for further analysis

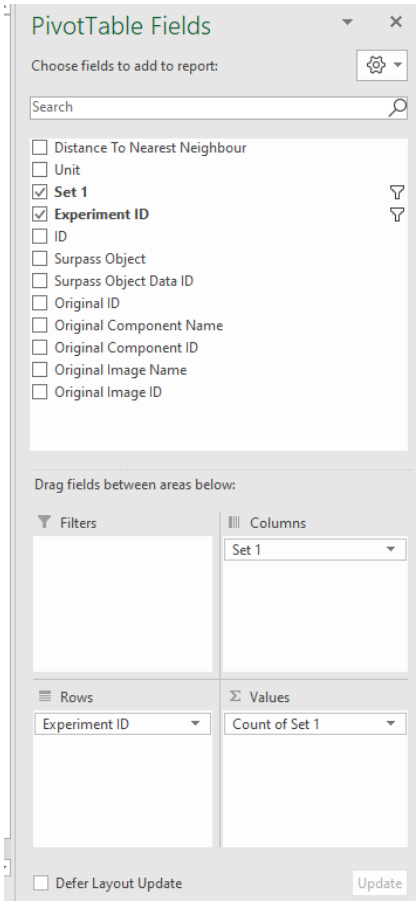
To get the counts per Class of Spots, we need to export the statistics to excel using the blue floppy disk icon. This was already done so no need to do it , this is just for your information.



You can explore in Excel file of the exported results  and see the following results:
in explorer under Learning Path images/6-Batch Colocalized Spots/ ScatterPlot

| Count of Set 1 | Set 1 | | | | |
|---|---|---|---|---|---|
| Experiment ID | Cyan Coloc Red | Cyan Not Coloc Red | Red Coloc Cyan | Red Not Coloc Cyan | Grand Total |
| Exp. 1 (images 1.1&1.2) | 16 | 2 | 16 | 2 | 36 |
| Exp. 2 (images 2.1&2.2) | 10 | 6 | 10 | | 26 |
| Grand Total | 26 | 8 | 26 | 2 | 62 |




Work in Excel:

1- The table was sorted by :
Level 1: “Original Image Name”, A to Z
Level 2: “Set 1”, A to Z

3- An extra column was manually added named “Experiment ID”

4- Insert a Pîvot Table

5-Pivot table fields beside

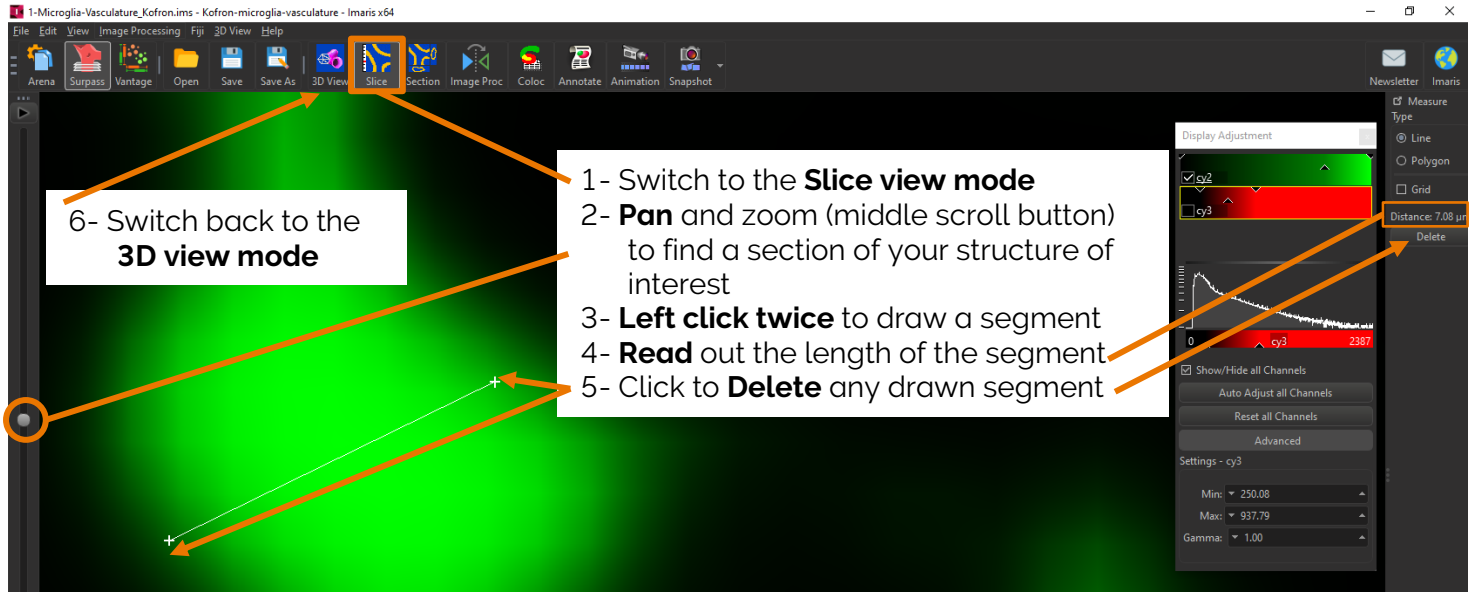
## More for later...about this topic:

You can have a look at the native image I used to create this exercise:
**6-FISH-Spot-Coloc-J-Chaumeil**
-Batch:

- Webinar: How to make your image analysis workflows more efficient?
- Tutorial video: Batch Pipeline for Image Processing and Object Detection
- Tutorial video: Batch Pipeline for Image Processing
- Tutorial video: Calculate Shortest Distance for Multiple Datasets at Once in the Batch Mode

-Colocalization tutorial and webinar

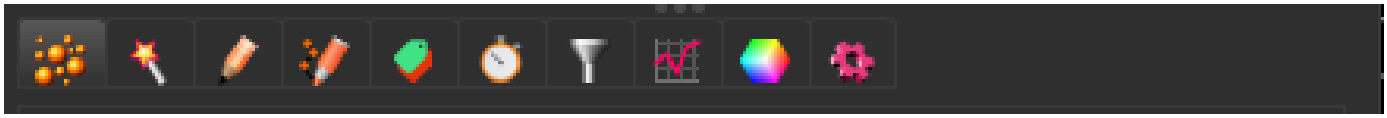
# Appendix1: How to quickly measure a distance:




**Back to Exercise 1** **Back to Exercise 2** **Back to Exercise 6**

# Appendix 2: Spots Property tabs

**Back to Exercise 1** **Back to Exercise 4**



**Settings :** Set Spots display

**Creation**:
- Rebuild Spots
- Creation parameters list that you can copy and paste in your e-notebook
- Save the used parameters to run them in a next similar image

**Edit**: useful for manual corrections, you can delete or add manually Spots, with Shift + Left Click:
- Pointing on the signal (cell with no Spot associated) to add a new Spot
- Pointing on an existing Spot to delete it

**Track Editor**: a line represents each track. Each line is composed of circles, representing objects, and line segments which connect a sequence of objects.
The first row/column of the track-editing table displays the timeline. Each number represents a time point in the series.
Track Editor has several features that help you modify your tracks

**Event:** Imaris 9.7 introduces the concept of "events" to allow alignment of time series data by "events" on the time axis**.**

**Classification**: you can manually label sets of Spots. The Label will appear in the statistics in the « Label » column.

**Filter**: you can select Spots according to any statistic and duplicate the selected sub-population to a new Spots object.

**Statistics:** all statistics calculated can be visualized and exported from this tab

**Color**: you can change the Spots rendering : colour, transparency, assign them a random colour (Object ID) and even colour code according to a statistic

**Tool**: gives access to installed Spots Xtensions. These are short editable scripts. You can add your own scripts (Matlab., Python, C++ …) or used existing Imaris ones (most of them are in Matlab)

**Back to Exercise 1** **Back to Exercise 4**

# Appendix 3: Imaris Tracking algorithms and options

**Back to Exercise 4**

**Brownian Motion**
This algorithm models the motion of each Spot as a Brownian motion. This model is appropriate if your Spots perform similar to a Brownian motion (the random movement of Spots with very frequent changes in direction and no discernible pattern).
The only prediction applied is that the Spot does not move further than the specified speed allows, so the maximum speed constrains the distance from one time-point to the next.

**Autoregressive Motion**
This algorithm models the motion of each Spot as an autoregressive AR1 process. This model is appropriate if your Spots perform any kind of continuous motion. The AR1 model looks back to one time-point, and predicts that the Spot will move again the same distance and in the same direction. The user-specified **Maximum Distance** value is the distance in which the Spot is allowed to deviate from the predicted position. Some changes in direction are tolerated because of the maximum distance parameter, and because AR1 does not remember farther back than one time-point.

**Autoregressive Motion Expert**
This algorithm models the motion of each Spot as an autoregressive AR1 process (see above). It is suitable for tracing of multiple objects with adjacent or crossing paths, especially if the neighboring objects have a stable (but different) intensity. In Autoregressive Motion Expert the algorithm called Total Cost determines the most appropriate connections for tracks by seeking the lowest Total Cost. In this case, the Total Cost algorithm combines Intensity Cost and Distance Cost functions (see Intensity Weight below).

**Connected Components:** For this algorithm, Spots in adjacent time-points are considered connected if the Spot spheres occupy some of the same space (the spheres would overlap if two time-points were merged into one). It compares the amount of overlaps between identified objects in the previous frame with those in the current frame. All the object with the overlap will be assigned the same track ID.
Connected Components and Lineage are the only algorithms that automatically handle lineage (spots that diverge). It will only work with images that have excellent time resolution, because the Spot cannot ever jump a distance larger than its own size.
NB: I nearly exclusively use this algorithm when I need object merges to be detected. It is the only algorithm which can do so.

**Lineage:** this model is intended for events such as cell division in which the splitting of objects is to be analyzed. This algorithm models the motion of each object as an autoregressive AR1 process (refer to Autoregressive Motion above). The object data is then compared so that only the objects that undergo splitting are connected, and assigned the same track ID. Objects that pass in close proximity, or overlap, are not associated. This is in contrast to connected components, where any objects that overlap are determined and considered connected.

**Max Distance:** disallows the connection between a spot and a candidate match if the distance between the predicted future position of the spot and the candidate position exceeds the defined maximum distance.

**Max Gap Size:** An object might fail to be segmented from some time points, which could lead to a Track breaking apart and the creation of Track fractions. Instead of creating track fragments, with gaps between undetected objects, the gap-closing algorithm creates tracks by linearly connecting objects associated with the same track. The time period in which the objects connection is automatically established is based on the value defined in the Max Gap Size value.
The Max Gap Size defines the maximum number of the consecutive time points, which are allowed to be missing in order for a track to continue.

**Back to Exercise 4**

# Appendix 4:Surfaces Property tabs

**Back to Exercise 2**

**Settings:** Set Surfaces display

**Creation**:

- Rebuild Surfaces
- Creation parameters list that you can copy and paste in your e-notebook
- Save the used parameters to run them in a next similar image

**Draw:** allows the user to semi-automatically create further Surface objects.

- **Add/Delete Shift + Left click to add** a Surface using either the Marching Cubes or Magic Wand technique
- **Contour**: to extract a 3D object by manually drawing the object contours on 2D slices. You can employ this method whenever simple threshold does not yield individual structure or to define manually drawn ROIs

**Edit**:

- For manual corrections, you can delete, cut, unify and split Surfaces
- Merge multiple Surfaces object from the list of object
- Duplicate Surfaces
- Create a new masked channel and/or a Distance Dransform channel.

The Distance Dransform channel is a new floating point image. This channel holds at each voxel the value for the shortest distance to the surface from the centre of that voxel. The values of the Distance Transform inside the surfaces will be negative

**Classification**: you can manually label sets of Surfaces. The Label will appear in the statistics in the « Label » column..

**Event:** Imaris 9.7 introduces the concept of “events” to allow alignment of time series data by “events” on the time axis**.**

**Filter**: you can select Surfaces according to any statistic and duplicate the selected sub-population to a new Surface object.

**Statistics** see next section

**Color**: you can change the Surface rendering : colour, texture, transparency, assign them a random colour (Object ID) and even colour code according to a statistic

**Tool**: gives access to installed Surfaces Xtensions. These are short editable scripts. You can add your own scripts (Matlab., Python, C++ …) or used existing Imaris ones (most of them are in Matlab)

**Back to Exercise 2**

# Appendix 5: Machine Learning Statistics Values

These features do not appear in the normal Imaris set of statistics values. For most purposes it should not be necessary to look at the list of statistics values computed for machine learning. The list of machine learning statistics is visible only on the **Settings tab** of the machine learning user interface.

**Imaris machine learning approach** is similar to: Ranzato, M., et al. "Automatic recognition of biological particles in microscopic images." *Pattern recognition letters* 28.1 (2007): 31-39. Ranzato et al. make use of multi-scale features invariant to shift and rotation as described originally by Koenderink and van Doorn, "Representation of local geometry in the visual system." *Biological cybernetics* 55.6 (1987): 367-375.

For classification of Spots Imaris computes Gaussian derivatives up to second order at a range of scales from 1 times the spot radius up to 8 times the spot radius. This creates a large set of shift and rotation invariant features that describe the local image intensity around a spot up to second order. In the list of machine learning statistics the names of these features all start with "**JET"**.
You can think of these features as describing **the local "intensity landscape"** of each channel at the position of the spot in terms of how high, how steep and how curved the landscape is.

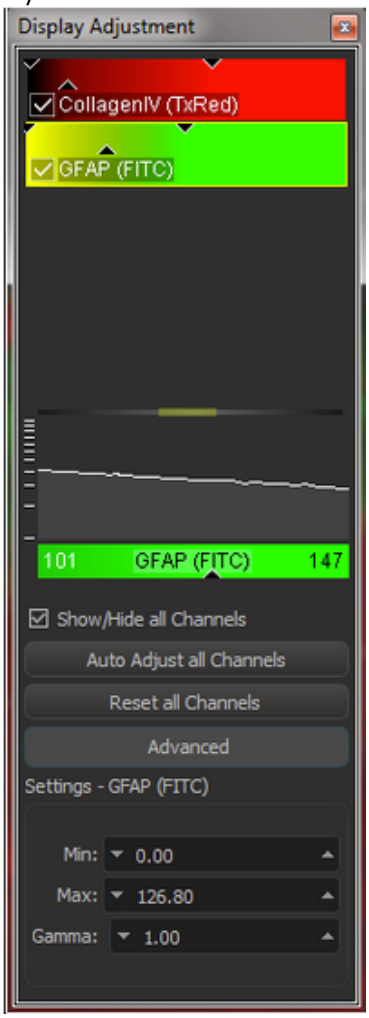
In addition to the Gaussian derivatives of the original channels Imaris also computes features that describe the local "**texture**" of an image around a spot. To do so it computes a binary image capturing the positions of local extrema in the original channel and from this binary image it computes the same Gaussian derivatives as for the original channel intensities. In the list of machine learning statistics the names of these features all start with "**TEX"**.

Both sets of features together provide a lot of useful features for a machine learning classifier.

**Back to Exercise 3** 

# Appendix 6: Display Adjustment window

The Display Adjustment options let you choose the channel visibility as well as improve the image display by concentrating on a limited color contrast range of voxels. Usually the color contrast values of the voxels stretch over a wide range (e.g. 0 - 255).




The Display Adjustment function lets you set an upper limit for maximum color and a lower limit for minimum color (i.e. black). The range between these two limits - represented by triangle

**Display Adjustment Dialog (one for each channel)**

Switch the individual channels on or off.

- Check or uncheck the required channel check-box to switch the channel visibility.

Change the channel parameters such as name, color and description.

- Click on the channel name to open the Image Properties. For a detailed description please refer to Section Menu Edit - Image Properties - Channels 1...n.

Show/Hide all Channels

- Check or uncheck to show or hide all channels as required.

Button Auto Adjust all Channels

- The auto function works as follows: the minimum value is set to the first mode (maximum) of the histogram. The maximum value is set to a value that is around the 99.8 percentile of the histogram which effectively disregards high intensity outliers.

Button Reset all Channels

- Click on the button to set the image back to the original values.

Button Advanced

- Click on the button to open the Advanced settings (see below).

**Advanced Settings**

Min and Max

- Enter direct values in the Min (lower limit for minimum color) and Max (upper limit for maximum color) fields.
- Alternatively drag in the display adjustment dialog the upper or lower handle of the adjustment line to adjust the Min and Max limits.

The effect of the change can be seen on the channels (channels appear brighter or darker).

Gamma

The default value of the gamma correction is 1 (the range between lower and upper limit is extrapolated in a linear mode to the full data set range). Enter a value below 1 and the linear mode is transferred to a nonlinear mode, the lower intensities appear brighter. The effect of the change is directly visible in the viewing area.

- Enter the value in the respective field.
- Alternatively click onto the middle triangle in the display adjustment dialog and drag it to the left to increase brightness/to the right to decrease brightness.

**Histogram**

A histogram is a statistical data analysis, representing linear voxel within an image of the selected channel.

**Change Channel Color**

- Click on the channel name to switch directly to the channel properties

**Back to Exercise 1** **Back to Exercise 2**